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Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) Title: MEDICAMENT WITH ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORS SUITABLE FOR TRANSDERMAL APPLICATION

(54) Bezeichnung: TRANSDERMAL APPLIZIERBARES ARZNEIMITTEL MIT ACE-HEMMERN

(57) Abstract

The invention concerns a transdermal system containing at least one ACE inhibitor.

(57) Zusammenfassung

Die Erfindung betrifft ein transdermales System mit einem Gehalt an mindestens einem Angiotensin Converting Enzyme-Hemmer.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

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Transdermal applizierbares Arzneimittel mit ACE-Hemmern

Die Langzeittherapie der Hypertonie mit Angiotensin Converting Enzyme-Hemmern (ACE-Hemmern) nimmt einen immer breiteren Raum ein. ACE-Hemmer sind bei guter Verträglichkeit für ihre zuverlässige Wirksamkeit bekannt. Die erste Substanz aus der Klasse der ACE-Hemmer, das Captopril, ist eine sehr hydrophile Substanz, die in unveränderter Form wirksam ist. Die orale Bioverfügbarkeit des Captoprils beträgt etwa 70 %. Neuere ACE-Hemmer, wie Enalapril, werden aus ihrer Vorstufe bei der Leberpassage in die wirksame Komponente Enalaprilat, das heißt die Säureform, metabolisiert. Wie Enalapril sind die ACE-Hemmer Ramipril, Cilacapril, Trandolapril, Benazepril oder Fosinopril lipophile Prodrugs der eigentlichen Wirkform der Dicarbonsäure. Durch die Veresterung jeweils einer Carboxylgruppe des jeweiligen ACE-Hemmers wird die Substanz lipophiler und dadurch für die orale Resorption günstiger. Die orale Bioverfügbarkeit dieser Prodrugs liegt jedoch immer niedriger als die des Captoprils. Sie beträgt beispielsweise für das Benazepril 28 % und für das Trandolapril ca. 40 bis 60 %. Nun ist es eine bekannte Tatsache, daß Substanzen mit geringer Bioverfügbarkeit sehr abhängig von der jeweili-

gen Metabolisierungsfähigkeit der Patienten sind. Das bedeutet, daß die resultierenden Plasmaspiegel einer sehr hohen Variation unterliegen. Die hohe Variation der Blutspiegel von ACE-Hemmern oder deren Wirkformen führt jedoch zu nicht kalkulierbaren Wirkungsverläufen. Um die Wirkung von ACE-Hemmern nun unabhängig von der metabolischen Lage der Patienten zu machen, wäre eine Arzneiform, die eine zuverlässige, reproduzierbare systemische Zufuhr der Wirkstoffe ermöglicht, wünschenswert. Die transdermale Applikation von Wirkstoffen führt zu einer Umgehung des hepatischen First-Pass-Metabolismus und damit zu einer Ausschaltung der Metabolisierungsvariationen der Leber. Gelänge es nun, ACE-Hemmer in Form ihrer Prodrugs oder Wirkformen transdermal systemisch verfügbar zu machen, könnte eine zuverlässigere gleichmäßige Wirkung erzielbar sein.

Aus WO-A1-9 323 019 ist bereits ein transdermales Reservoir-System mit einem Gehalt an einem ACE-Hemmer und

(a) einer undurchlässigen Abdeckschicht (Backing Layer),
(b) einem schichtartigen Element mit Hohlraum,
(c) einem die Wirkstoffabgabe steuernden Mittel (claim 1) und
(e) einer abziehbaren Deckschicht (Release Liner) auf Papierbasis (Seite 12 Zeilen 7/8) bekannt.

Transdermale Systeme mit einem Gehalt an einem ACE-Hemmer werden ferner in EP-A2-0 439 430 (Reservoir-TTS) und EP-A2-0 468 875 (Matrix-TTS) beschrieben, wobei nach EP-A2-0 468 875 Silikon-Elastomere als Matrixmaterial verwendet werden.

Aufgabe der vorliegenden Erfindung ist es, ein System für die transdermale Zufuhr von ACE-Hemmern vorzusehen, insbesondere von Ramipril, Trandolapril und/oder deren therapeutisch wirksamen Salzen, das gegenüber dem Stand der Technik verbessert ist.

Insbesondere ist es Aufgabe der Erfindung, ein System für die transdermale Zufuhr von ACE-Hemmern vorzusehen, mit dem sich eine Wirksamkeit von bis zu etwa einer Woche erreichen läßt, so daß für etwa eine Woche eine kontinuierliche Abgabe an Wirkstoff und ein therapeutisch wirksamer Plasmaspiegel erreicht werden können, beispielsweise von mehr als 0,5 ng Trandolapris/ml.

Dazu wird erfindungsgemäß ein transdermales System mit einer Matrix auf Basis von Polyisobutylen oder Butylkautschuk und mit einem Gehalt an mindestens einem ACE-Hemmer vorgesehen. Erfindungsgemäß wurde überraschenderweise festgestellt, daß lipophile ACE-Hemmer oder deren Wirkformen, die die menschliche Haut nur schwer permeieren können, mit Hilfe eines transdermal applizierbaren Arzneimittels mit einer Polyisobutylenmatrix oder Butylkautschukmatrix die Haut gut durchdringen können und einen zuverlässigen, kontinuierlichen Blutspiegel erzeugen.

Erfindungsgemäß kann eine Abgaberate des Wirkstoffs aus beispielsweise einer Polymermatrix von 0,01 bis 0,1 mg Wirkstoff/cm^2 24 h und insbesondere 0,025 bis 0,050 mg Wirkstoff/cm^2 24 h erreicht werden, so daß ein erfindungsgemäßes transdermales System eine Plasmakonzentration an Wirkstoff in einer therapeutisch wirksamen Menge bietet. Beispielsweise läßt sich für Trandolapril eine therapeutisch wirksame Konzentration im Blut von mehr als etwa 0,5 ng/ml erzielen.

Der Fachmann ist mit geeigneten Matrizes aus Polyisobutylen oder Butylkautschuk vertraut; vgl. beispielsweise Higgins et al. in Satas, Handbook of Pressure Sensitive Adhesive Technology, 14 : 374 etc., Butyl Rubber and Polyisobutylene; Van Nostrand Reinhold, New York.

Bei dem erfindungsgemäßen transdermalen System kann der ACE-Hemmer in einer Konzentration von mindestens 5 Gew.-% und insbeson-

dere in einer Konzentration von 10 bis 20 Gew.-% (bezogen auf die Matrix) vorliegen.

Der ACE-Hemmer kann dabei als Prodrug oder als Wirkform eingesetzt werden.

Als Beispiele für ACE-Hemmer seien Ramipril, Trandolapril und/oder deren Wirkformen (Säureformen) sowie deren therapeutisch wirksame Salze genannt.

Das erfindungsgemäße transdermale System kann einem Permeationsförderer umfassen, beispielsweise 2-Octyldodecanol (Eutanol G).

Bei den erfindungsgemäßen Transdermalsystemen können unterschiedliche Formen Anwendung finden, beispielsweise membran- oder matrixkontrollierte Systeme.

So kann es sich beim erfindungsgemäßen transdermalen System um ein Pflaster mit einem Reservoir handeln (Pflaster vom Reservoirtyp).

Gemäß einer speziellen Ausführungsform kann ein derartiges Pflaster mit Reservoir durch

(a) eine undurchlässige Abdeckschicht (Backing Foil),
(b) ein schichtartiges Element mit Hohlraum,
(c) eine microporöse oder semipermeable Membran,
(d) eine selbstklebende Schicht (Haftschicht) und
(e) gegebenenfalls eine abziehbare Deckschicht (Release Liner)

gekennzeichnet sein.

Dabei kann das schichtartige Element mit Hohlraum durch die Abdeckschicht und die Membran gebildet werden.

Die microporöse oder semipermeable Membran kann aus einem inerten Polymeren, beispielsweise Polypropylen, Polyvinylacetat oder Silikon bestehen.

Gemäß einer weiteren speziellen Ausführungsform der Erfindung kann das Pflaster vom Reservoir-Typ durch

(a) eine undurchlässige Abdeckschicht (Backing Foil),
(b) einen offenporigen Schaum, einen geschlossenporigen Schaum, eine gewebeartige Schicht oder eine vliesartige Schicht als Reservoir,
(c) sofern die Schicht gemäß (b) nicht selbstklebend ist, eine selbstklebende Schicht (Haftschicht) und
(d) gegebenenfalls eine abziehbare Deckschicht (Release Liner)

gekennzeichnet sein.

Das Reservoir kann also beispielsweise durch einen Hohlraum oder auf andere Weise gebildet werden. Das Reservoir ist dabei mit dem Wirkstoff/Gemisch der Hilfsstoffe gefüllt. Für die Aufnahme des Wirkstoffs im Reservoir kann auf den Stand der Technik für Reservoir-Systeme verwiesen werden. Nach Abziehen der Abdeckfolie (Schutzfolie) und Aufkleben des Pflasters auf die Haut permeiert der Wirkstoff mit den Hilfsstoffen (durch die gegebenenfalls vorgesehene Membran) durch die Klebeschicht in die Haut.

Sofern eine Membran vorgesehen ist, kann sie je nach Porenweite eine die Freisetzung des Wirkstoffs kontrollierende Wirkung oder auch keinen Einfluß auf die Wirkstofffreisetzung aus dem System haben.

Wird das Reservoir durch einen offenporigen Schaum, einen geschlossenporigen Schaum, eine gewebeartige Schicht oder eine vliesartige Schicht vorgesehen, so liegen Wirkstoff/Gemisch der Hilfsstoffe aufgesogen bzw. fein verteilt vor. In diesem Fall

kann eine microporöse oder semipermeable Membran fehlen, die das Reservoir bildende Schicht selbstklebend sein oder (sofern das nicht der Fall ist) eine selbstklebende Schicht (Haftschicht) tragen.

Gemäß einer speziellen Ausführungsform kann das erfindungsgemäße transdermale System durch

(a) eine undurchlässige Abdeckschicht (Backing Foil),
(b) eine Matrixschicht für den Wirkstoff,
(c) (sofern die Schicht gemäß (b) nicht selbstklebend ist) eine wirkstoffdurchlässige Haftklebeschicht und
(d) gegebenenfalls eine abziebare Deckschicht (Release Liner)

gekennzeichnet sein.

Als Matrix kann erfindungsgemäß ein selbstklebender Polyisobutylenkleber verwendet werden.

Nachstehend wird die Erfindung durch Beispiele näher erläutert.

Beispiele 1 bis 5

Es wird ein transdermales therapeutisches System (TTS) vom Matrixtyp vorgesehen, das beispielsweise durch die folgende Zusammensetzung gekennzeichnet ist.

Matrix:	Polyisobutylenkleber (MA24 von Adhesive Research Inc., Glen Rock, Pennsylvania, USA)
Abdeckfolie:	Polyesterfolie (Hostaphan RN 19)
Abziehfolie:	Polyesterfolie (Gelroflex PET 75 µm 1-S) oder beschichtete Papierfolie (Gelrolease 603/100 DRS)

Matrixbestandteile:	Trandolapril	10 Gew.-%
	Eutanol G	5 Gew.-%
	Polyisobutylenkleber (Trockenmasse)	85 Gew.-%

Vergleichsbeispiel 1

Hier wird anstatt eines Polyisobutylenklebers ein Silikonkleber (BIO PSA X7 4302) verwendet.

Die erhaltenen Ergebnisse sind der folgenden **Tabelle** zu entnehmen.

Eine Gegenüberstellung von Beispiel 1 und Vergleichsbeispiel 1 zeigt, daß die Wirkstoffaufgabe bei dem erfindungsgemäßen System über einen Zeitraum von 20 Tagen konstant bleibt, während sie beim Vergleichsbeispiel 1 drastisch abfällt.

Anwendungsbeispiel 1

In einer in-vivo-Vergleichsstudie eines erfindungsgemäßen TTS mit einer oralen Gabe von Trandolapril (Kapsel) wurde bei 6 gesunden Probanden das pharmakokinetische Verhalten für TTS-Applikation geprüft. Dabei wurden im offenen 2-Perioden-cross-over-design die TTS über einen Zeitraum von 7 Tagen (1 TTS 4 Tage, anschließend 1 TTS 3 Tage) appliziert und im Vergleich 7 Tage lang 1 Kapsel à 2 mg Trandolapril täglich appliziert. Blutproben wurden nach folgenden Zeiten genommen: -0,5; 0; 2; 4; 6; 8; 10; 12; 24; 48; 72; 96; 98; 100; 102; 104; 106; 108; 120; 132; 144; 156; 168 h nach Applikation.

Die pharmakokinetischen Ergebnisse zeigen, daß das TTS ein grundsätzlich anderes Blutspiegelprofil aufweist als die Kapseln. Im Gegensatz zur Kapsel wird ein über den jeweiligen Applikationszeitraum von 3 bzw. 4 Tagen konstanter Blutspiegel er-

zielt, was therapeutisch auch wünschenswert ist. Nach oraler Gabe steigt die Blutkonzentration schnell an, und zwar werden innerhalb 2 h ca. 5 ng/ml erreicht. Die Elimination erfolgt mit einer Halbwertzeit von ca. 24 h. Im Vergleich dazu ist der Blutspiegelverlauf nach TTS-Gabe gleichmäßiger. In den ersten 4 Tagen der Anwendung ist ein gleichmäßiger leichter Anstieg des Blutspiegels von ca. 0,3 ng/ml nach 6 h auf ca. 1 ng/ml nach 96 h zu beobachten. Nach Wechsel des TTS nach 96 h steigen die Blutspiegel in der zweiten Applikationsperiode nur noch unwesentlich an (**Figur 1**). Damit kommt das Blutspiegelprofil nach Gabe der TTS dem therapeutischen Ideal von konstanten Blutspiegeln während der Behandlung sehr nahe. Unerwünschte Blutspiegelspitzen, die mit unerwünschten Nebenwirkungen wie plötzlichem Blutdruckabfall verbunden sein können, werden sicher vermieden.

Beispiel	Kleber	Wirkstoff: Trandolapril	Permeations-förderer	Wirkstoffabgabe [mg/cm^2] Tag ab Herstellung	Haut-permeation [24 h]	Diss. [6 h]	Kristal-lisation
		[%]	[%]				
B 1	Polyiso-butylen	3	0	4	0.013		keine
				7		0.008	
				20		0.008	
B 2		5	0	4	0.014		keine
				7		0.009	
				20		0.011	
B 3		10	0	4	0,027		keine
				7		0.019	
				20		0.019	
				37	0.023		
B 4		10	10 Cetiol V	13	0.044		keine
B 5		10	5 Eutanol G	8	0.061		keine
VB 1	Silikon	3	0	0	0.065		keine
				15	0.030		keine
				26		0.076	
				33	0.017		

Patentansprüche

1. Transdermales System mit einer Matrix auf Basis von Polyisobutylen oder Butylkautschuk und mit einem Gehalt an mindestens einem Angiotensin Converting Enzyme-Hemmer (ACE-Hemmer).

2. Transdermales System nach Anspruch 1, dadurch **gekennzeichnet**, daß der ACE-Hemmer in einer Konzentration von mindestens 5 Gew.-% und insbesondere in einer Konzentration von 10 bis 20 Gew.-% (bezogen auf die Matrix) vorliegt.

3. Transdermales System nach Anspruch 1 oder 2, dadurch **gekennzeichnet**, daß der ACE-Hemmer als Prodrug oder als Wirkform vorliegt.

4. Transdermales System nach einem der vorhergehenden Ansprüche, **gekennzeichnet** durch Ramipril, Trandolapril und/oder deren Wirkformen (Säureformen) und/oder deren therapeutisch wirksame Salze als ACE-Hemmer.

5. Transdermales System nach einem der vorhergehenden Ansprüche, dadurch **gekennzeichnet**, daß das transdermale System einen Permeationsförderer umfaßt, insbesondere Eutanol G.

6. Transdermales System nach einem der vorhergehenden Ansprüche, dadurch **gekennzeichnet**, daß das transdermale System ein Pflaster mit einem Reservoir ist (Pflaster vom Reservoir-Typ).

7. Transdermales System nach Anspruch 6, **gekennzeichnet** durch
(a) eine undurchlässige Abdeckschicht (Backing Foil),
(b) ein schichtartiges Element mit Hohlraum,
(c) eine microporöse oder semipermeable Membran,
(d) eine selbstklebende Schicht (Haftschicht) und
(e) gegebenenfalls eine abziehbare Deckschicht (Release Liner).

8. Transdermales System nach Anspruch 7, dadurch **gekennzeichnet**, daß das schichtartige Element mit Hohlraum durch die Abdeckschicht und die Membran gebildet wird.

9. Transdermales System nach Anspruch 7 oder 8, dadurch **gekennzeichnet**, daß die Membran aus einem inerten Polymeren besteht, insbesondere Polypropylen, Polyvinylacetat oder Silikon.

10. Transdermales System nach Anspruch 6, **gekennzeichnet** durch

(a) eine undurchlässige Abdeckschicht (Backing Foil),
(b) einen offenporigen Schaum, einen geschlossenporigen Schaum, eine gewebeartige Schicht oder eine vliesartige Schicht als Reservoir,
(c) sofern die Schicht gemäß (b) nicht selbstklebend ist, eine selbstklebende Schicht (Haftschicht) und
(d) gegebenenfalls eine abziehbare Deckschicht (Release Liner).

11. Transdermales System nach einem der Ansprüche 1 bis 5, **gekennzeichnet** durch

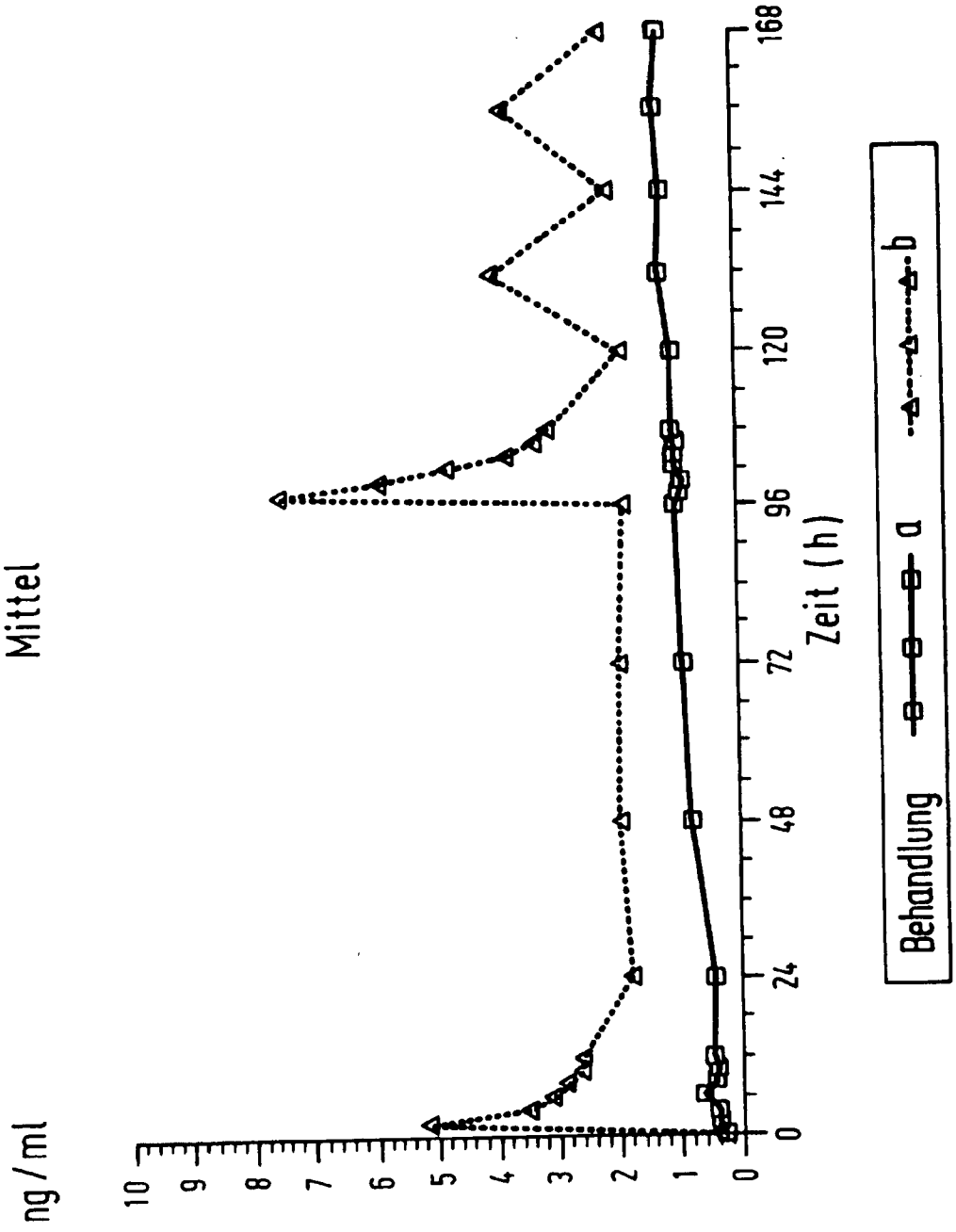
(a) eine undurchlässige Abdecksicht (Backing Foil),
(b) eine Matrixschicht für den Wirkstoff,
(c) (sofern die Schicht gemäß (b) nicht selbstklebend ist) eine wirkstoffdurchlässige Haftklebeschicht und
(d) gegebenenfalls eine abziehbare Deckschicht (Release Liner).

12. Transdermales System nach Anspruch 11, **gekennzeichnet** durch einen selbstklebenden Polyisobutylenkleber als Matrix.

13. Transdermales System nach einem der Ansprüche 7 bis 12, dadurch **gekennzeichnet**, daß die Abdeckschicht (Backing Foil) aus Polyester, Polypropylen, Polyethylen oder Polyurethan gebildet ist.

14. Transdermales System nach einem der Ansprüche 7 bis 13, dadurch **gekennzeichnet**, daß die abziehbare Deckschicht (Release Liner) aus Polyester, Polypropylen oder beschichtetem Papier (Papier mit Beschichtung) gebildet ist, insbesondere mit einer Silikon- und/oder Polyethylen-Beschichtung.

15. Transdermales System nach einem der Ansprüche 13 oder 14, **gekennzeichnet** durch eine Abdeckschicht (Backing Foil) und/oder abziehbare Deckschicht (Release Liner) mit einer Dicke im Bereich von 5 bis 100 μm.


Mittel
ng/ml
10
9
8
7
6
5
4
3
2
1
0
0
24
48
72
96
120
144
168
Zeit (h)
Behandlung
a
b

INTERNATIONAL SEARCH REPORT

National Application No
PCT/EP 96/01402

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/70 A61K38/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 425 837 (NITTO ELECTRIC INDUSTRIAL CO LTD) 23 October 1991 see page 3, line 36 - line 44 see page 6, line 58 - page 7, line 4 see page 10; example 1	1-3,6
A	EP,A,0 439 430 (CIBA GEIGY AG) 31 July 1991 cited in the application see page 4, line 28 - line 40 see claims 1,2	1
A	WO,A,93 23019 (SRI INTERNATIONAL) 25 November 1993 cited in the application see page 7, line 26 - line 29	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1996	22. 08. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Boulois, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 96/01402

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1 - 3
because they relate to subject matter not required to be searched by this Authority, namely:

A medicament cannot be characterised in terms of a pharmacological activity such as "ACE inhibition" and must instead be characterised in terms of its structure.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No
PCT/EP 96/01402

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-425837	08-05-91	JP-A-	3122089	24-05-91
		JP-B-	7074116	09-08-95
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		CA-A-	2034516	23-07-91
		JP-A-	4297417	21-10-92
WO-A-9323019	25-11-93	NONE		

INTERNATIONALER RECHERCHENBERICHT

nationales Aktenzeichen
PCT/EP 96/01402

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 6 A61K9/70 A61K38/55

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 6 A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	EP,A,0 425 837 (NITTO ELECTRIC INDUSTRIAL CO LTD) 23.Oktober 1991 siehe Seite 3, Zeile 36 - Zeile 44 siehe Seite 6, Zeile 58 - Seite 7, Zeile 4 siehe Seite 10; Beispiel 1 ---	1-3,6
A	EP,A,0 439 430 (CIBA GEIGY AG) 31.Juli 1991 in der Anmeldung erwähnt siehe Seite 4, Zeile 28 - Zeile 40 siehe Ansprüche 1,2 ---	1
A	WO,A,93 23019 (SRI INTERNATIONAL) 25.November 1993 in der Anmeldung erwähnt siehe Seite 7, Zeile 26 - Zeile 29 -----	1

☐ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

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Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
7.August 1996	22. 08. 96

Name und Postanschrift der Internationale Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Boulois, D

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/EP 96/01402

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 1 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

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2. [ ] Ansprüche Nr.
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3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 2 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche der internationalen Anmeldung.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Internationale Recherchenbehörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

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4. [ ] Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

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[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

[ ] Die Zahlung zusätzlicher Gebühren erfolgte ohne Widerspruch.

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffen...ungen, die zur selben Patentfamilie gehören

nationales Aktenzeichen
PCT/EP 96/01402

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
EP-A-425837	08-05-91	JP-A-	3122089	24-05-91
		JP-B-	7074116	09-08-95
		DE-D-	69011619	22-09-94
		DE-T-	69011619	12-01-95
		US-A-	5269875	14-12-93
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		AU-B-	6934691	25-07-91
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— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

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WO 01/00247 A1

(54) Title: PEPTIDE-LIPID CONJUGATES, LIPOSOMES AND LIPOSOMAL DRUG DELIVERY

(57) Abstract: Peptide-lipid conjugates are incorporated into liposomes so as to selectively destabilize the liposomes in the vicinity of target peptidase-secreting cells, and hence, to deliver the liposomes to the vicinity of the target cells, or directly into the cells. The liposomes can thus be used to treat mammals for diseases, disorders or conditions, e.g., tumors, microbial infection and inflammations, characterized by the occurrence of peptidase-secreting cells.

PEPTIDE-LIPID CONJUGATES, LIPOSOMES AND LIPOSOMAL DRUG DELIVERY

Cross-References to Related Applications.

This application is a continuation-in-part ("CIP") of our copending U.S. application, Serial No. 09/168,010, filed October 7, 1998, which is a division of U.S. application Serial No. 08/950,618, filed October 15, 1997, which is a CIP that claims the benefit of provisional application U.S. Serial No. 60/027,544, filed October 15, 1996.

This application is also a CIP of our copending U.S. application Serial No. 09/032,059, filed February 27, 1998, which is a CIP that claims the benefit of provisional application U.S. Serial No. 60/039,183, filed February 27, 1997.

Field of the Invention

Peptide-lipid conjugates are incorporated into liposomes so as to localize delivery of the liposomes' contents to the vicinity of target cells.

Background of the Invention

Liposomes have been widely used as carriers to deliver a variety of therapeutic and diagnostic agents into cells. Encapsulation of active agents in liposomes protects the agents from premature degradation, and ameliorates side effects resulting from administration of the agents to animals (for a review, see, e.g., A. Bangham, 1992; M. Ostro, 1987; and, M. Ostro and P. Cullis, 1989). However, the efficiency of liposomal drug delivery has heretofore been constrained by the lack of a means of inducing liposomes to preferentially release their contents in the vicinity of, or into, target cells. This invention provides such a means, by incorporating peptide-lipid conjugates into liposomes and then contacting cells with these liposomes.

The lipid portion of the peptide-lipid conjugate is a phosphatidylethanolamine ("PE"). Many of these lipids ordinarily do not organize into bilayers at neutral pH, instead forming hexagonal (H_{II})-phase structures in aqueous environments which tend to destabilize the bilayers of liposomes into which the lipids have been incorporated.

These same structures can also enhance the liposomes' fusogenicity (Verkleij, 1984; Cullis & de Kruijff, 1979; Ellens et al., 1989). Conjugation of a peptide to the PE stabilizes the PE in a bilayer conformation and hence, allows the conjugated lipid to be stably incorporated into liposome bilayers. However, once the peptide is cleaved, e.g., in the vicinity of peptidase-secreting cells, the lipid then resumes its nonbilayer-preferring conformation, in which it tends to destabilize the same liposome bilayers. In addition, the peptide linker could bind a blocking group, such as a polyethyleneglycol (PEG), such that it may act to sterically hinder fusion

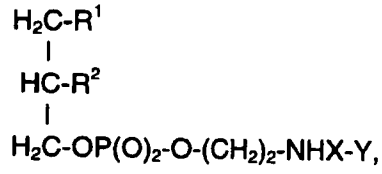
The peptide portion of the peptide-lipid conjugate is any of those peptides having amino acid sequences that are recognized and cleaved by any of the various peptidases secreted by mammalian cells, e.g., at sites of inflammation and tumor metastases (see, e.g.: Aimes and Quigley, 1995; Fosang et al., 1994; Froelich et al., 1993; Knauper et al., 1996; Liotta et al., 1991; Moehrle et al., 1995; Nagase et al., 1994; Nakajima et al., 1979; Odake et al., 1991; Palmieri et al., 1989; Pei et al., 1994; Prechel et al., 1995; Yamashita et al., 1994). Neither linkage of peptidase-cleavable peptides nor the incorporation of such peptides into liposomes, let alone for the purpose of promoting controlled liposome destabilization, has previously been described.

Vogel et al. (1993l and Subbaro et al. (1967) both covalently linked peptides to PEs; however, these peptide-lipids are not described therein as being cleavable by cell-secreted peptidases. Rather, the peptide-modified lipids of these documents are pH sensitive, adopting an alpha-helical conformation in low pH endosomal environments. Kirpotin et al. modified distearoyl phosphatidylcholine ("DSPE") by the attachment thereto of methoxypoly(ethylene glycol) ("mPEG") to DSPE on the amino group; liposomes containing mPEG-modified DSPE were stable in solution until thiolytic cleavage and removal of the mPEG moiety. Kirpotin does not describe the peptide-based modification of PEs, let alone with peptidase-cleavable peptides.

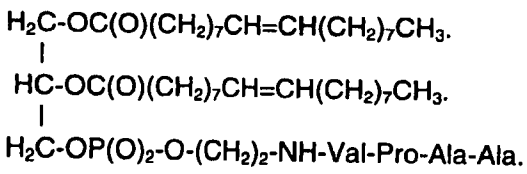
SUMMARY OF THE INVENTION

This invention provides a means of delivering and localizing the contents of liposomes to the vicinity of cells in a controlled manner, by conjugating certain peptides to phosphatidylethanolamines, and then incorporating these conjugated lipids into liposomes. The resulting liposomes are stable so long as the peptide remains conjugated to the lipid. However, once the peptide portion of the conjugate is cleaved from the lipid, by the action of cell-secreted or associated peptidases, the liposomes tend to destabilize, so as to release their contents in the vicinity of, or into, the secreting cells. Delivery of the liposomes' contents is thus targeted to the peptidase-secreting cells.

Peptide-lipid conjugates of this invention have the formula:



wherein: each of R^1 and R^2 is an acyl chain, X is a single bond or an acyl chain, and Y is a peptidase-cleavable peptide. The acyl chains are preferably oleic acid chains, X is preferably a single bond, and the peptide preferably contains the amino acid sequence Ala-Ala- (SEQ ID NO: 1); more preferably N-Acetyl-Ala-Ala- (SEQ ID NO: 2), or Ala-Ala-Pro-Val- (SEQ ID NO:3); and most preferably, N-methoxysuccinyl-Ala-Ala-Pro-Val (SEQ ID NO:4). Accordingly, the peptide-lipid conjugate preferably has the formula:



The liposomes' lipid component can be entirely composed of the peptide-lipid conjugate, or can comprise one or more additional lipids. Such additional lipids include, without limitation, any of the types of lipids, e.g., phospholipids, glycolipids and sterols,

which may be used in the preparation of liposomes. Most preferably, the liposome of this invention comprises a peptide-lipid conjugate and the positively charged synthetic lipid 1-N,N-dimethylamino dioleoyl propane (DODAP).

Controlled delivery with the liposomes of this invention can be used to deliver the liposomal drugs and/or bioactive agents in vitro or in vivo, for example, in the treatment of mammals afflicted with various diseases, disorders or conditions, e.g., cancers, amenable to treatment with the bioactive agent associated with the liposome.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structure of *N*-Ac-AA-DOPE and postulated scheme of conversion to DOPE by enzymatic cleavage.

Figure 2. TLC determination of protease mediated cleavage of *N*-Ac-AA-DOPE. *N*-Ac-AA-DOPE SUVs were incubated with elastase or proteinase K (1 mg enzyme/100 nmol lipid/0.1 ml buffer) overnight at 37°C (results of elastase-mediated cleavage are depicted in Fig. 2A, and results of proteinase K-mediated cleavage are depicted in Fig. 2B). Lipid was collected and separated by TLC. Lipid spots were developed as described hereinbelow. Lane 1, *N*-Ac-AA-DOPE without enzyme; lane 2, *N*-Ac-AA-DOPE with enzyme treatment; lane 3, DOPE from stock solution.

Figure 3. ^{31}P NMR spectrum of N-Ac-alanyl-alanyl-dioleoyl phosphatidylethanolamine, DOPE and enzyme-treated liposomes.
Panel A., Pure N-Ac-ala-ala-DOPE
Panel B, Multilamellar liposomes, prepared as described below, comprising pure N-Ac-ala-ala-DOPE (2 umole total phospholipid) incubated in 200 ul of 154 mM NaCl, 10 mM TES, 0.1 mM EDTA at pH 7.4 and 37 degrees C for a total of two hours in the presence of 1 mg of proteinase K, as described in Example 2; and
Panel C, pure DOPE.

Figure 4. Proteinase K mediated cleavage of *N*-Ac-AA-DOPE. DOTAP/*N*-Ac-AA-DOPE (1:1) SUVs were incubated with or without elastase, proteinase K, or heat

inactivated proteinase K (95°C, 1 hour) at a concentration of 1 mg protease/100nmol lipid/0.1 ml buffer overnight at 37°C. Lipid was collected and analyzed by HPLC. The *N*-Ac-AA-DOPE peak was quantitated and the amount of cleavage was calculated as a percentage of the starting lipid. The bar indicates the range of data from several experiments.

Figure 5. TLC determination of the cleavage of MeO-suc-ala-ala-pro-val-DOPE. Fig. 5A. HLE (Human leukocyte elastase) dose titration : Lane 1/ 0 ug HLE/100nmol lipid; 2/ 5 ug HLE/100nmol lipid; 3/ 10 ug HLE/100nmol lipid; 4/ pure DOPE, 20ug; 5/ 20 ug HLE/100nmol lipid; 6/ 40 ug HLE/ 100nmol lipid; 7/ 40 ug proteinase K/100nmol lipid; and lane 8/ pure DOPE, 20ug;
Fig. 5B. Kinetics of HLE cleavage of MeO--suc-AAPV-PE 1/ without protease; 2/ 1 hour, 5 ug HLE/50nmol lipid; 3/ 2 hours, 5 ug HLE/50nmol lipid; 4/ pure DOPE, 20ug; 5/ 4 hours, 5 ug HLE/50nmol lipid; 6/ overnight, 5 ug HLE/50nmol lipid; 7/ pure DOPE, 20ug.
Fig. 5C Cleavage of MeO-suc-AAPV-PE by human neutrophil granule proteins: Lane 1/ without protease; 2/ 5 ug HLE/50nmol lipid; 3/ 2.5 ug granule proteins/100nmol lipid; 4/ pure DOPE, 20ug; 5/ 5 ug granule proteins/100nmol lipid; 6/ 10 ug granule proteins/100nmol lipid; 7/ 20 ug granule proteins/100nmol lipid; 8/ pure DOPE, 20ug.

Figure 6. Quantitation of elastase-mediated cleavage of MeO-suc-AAPV-DOPE to DOPE by ^{31}P-NMR. DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) freeze-thaw/extrusion vesicles were incubated with 0; 0.5; 1; 2; and 5 micrograms of elaste/100 nmol lipid. Samples were incubated for 2 hours at 37ºC, after which liposomes were pelleted by ultracentrifugation. Liposomes were solubilized and monitored by ^{31}P-NMR. Solid line: Percentage of total MEO-suc-AAPV-DOPE converted to DOPE; dotted line; % of expected MeO-suc-AAPV-DOPE on outer monolayers converted to DOPE assuming unilamellar vesicles, i.e., result multiplied by 2.

Figure 7. Effect of proteolytic enzymes on cleavage of N-Ac-ala-ala-PE. X-axis: % lipid cleaved; Y-axis: enzyme used (from top to bottom): S. caespitosus; S. griseus ("pronase"); peptidase; chymotrypsin; trypsin; protease type I; proteinase K; no enzyme added. Solid bars: experiment 1, using enzyme at a concentration of 0.5 mg

enzyme/100 nmole liposomes. Open bars: experiment 2, using enzyme at a concentration of 1 mg enzytme/10 nmole liposomes. Liposomes were sonicated SUVs composed of a 1:1 mixture of DOTAP/N-Ac-ala-ala-DOPE (molar ratio).

Figure 8. Determination of optimal liposomal composition. Liposomes were prepared having the molar ratios of DOTAP, *N*-Ac-AA-DOPE, PE. shown in the Figure. One mol % *N*-NBD-PE and *N*-Rho-PE fluorescent probes were included in all preparations. Liposomes were mixed with unlabeled PE/PS or PC/PS (80/20 mol%; 1:10 effector:acceptor ratio; 60uM total lipid) (Fig. 8A) or $2x10^8$ RBC ghosts (Fig. 8B) at 37°C for 1 hour. Lipid mixing was calculated as the percentage of *N*-NBD-PE FDQ relative to maximal FDQ, as determined by detergent addition. Binding of liposomes to RBC ghosts was quantitated after washing cells with buffer, by calculating the amount of *N*-Rho-PE fluorescence associated with the cell pellet relative to the total input fluorescence.

Figure 9. Concentration and time dependence of proteinase K activity. Activation of fusion: DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes (100nmol) containing fluorescent membrane probes were incubated in 0.1ml buffer at 37°C either overnight (Fig. 9A) with given amounts of proteinase K or (Fig. 9B) with 1 mg proteinase K for given times. 10nmol aliquots were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10 effector:acceptor ratio), after which lipid mixing was determined. *N*-Ac-AA-DOPE cleavage: unlabeled DOTAP/*N*-Ac-AA-DOPE (1:1 mol ratio) liposomes were treated identically as for fusion activation, after which lipid was extracted and analyzed by HPLC, and the amount of cleavage was calculated as described previously.

Figure 10. Elastase and proteinase K mediated activation of liposomal fusion. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes containing fluorescent membrane probes were pretreated with human leukocyte elastase or proteinase K (1 mg protein/100 nmol lipid/0.1 ml buffer) overnight at 37°C. 10nmol aliquots were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10

effector:acceptor ratio) for 60 min at 37°C. Lipid mixing was determined by monitoring *N*-NBD-PE FDQ.

Figure 11. Requirement for active proteinase K for DOTAP/*N*-Ac-AA-DOPE/PE liposome fusion activation with PS/PE liposomes. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes (100nmol) containing fluorescent membrane probes were pretreated with or without 1 mg of proteinase K or heat inactivated proteinase K (1 hour, 95°C) overnight at 37°C in 0.1ml buffer. 10nmol aliquots were incubated with unlabeled PE/PS acceptor liposomes (80/20 mol%; 1:10 effector:acceptor ratio), after which lipid mixing was determined. Prot K carryover = effect of residual proteinase K carried over to incubation mixture with PE/PS liposomes was monitored by incubating untreated DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes with PE/PS liposomes in presence of freshly added proteinase K equivalent to the expected transferred amount.

Figure 12. Activation of DOTAP/*N*-Ac-AA-DOPE/PE liposomes by proteinase K for fusion with RBC ghosts. DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes (100nmol) containing fluorescent membrane probes were incubated overnight at 37°C with or without 1 mg of proteinase K in 0.1 ml buffer. 10nmol aliquots of DOTAP/*N*-Ac-AA-DOPE/PE liposomes as well as DOTAP/PE (20/80 mol%) liposomes were incubated with $1x10^8$ RBC ghosts in buffer containing 0.5mM PMSF for 30min at 37°C, after which lipid mixing was determined. Effect of transferred proteinase K on lipid mixing was monitored by incubating untreated liposomes with RBC ghosts in presence of equivalent amount of proteinase K (prot K control).

Figure 13 DOTAP/*N*-Ac-AA-DOPE/PE liposome with RBC ghosts: continuous kinetics of lipid mixing. 10nmol of DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes incubated (a) with or (b) without proteinase K overnight at 37°C were added to a cuvette containing 2 ml buffer with 0.5mM PMSF under continuous stirring and 37°C conditions. *N*-NBD-PE fluorescence recording was initiated and $1x10^8$ RBC ghosts were added at 30 sec. (c) Effect of carryover proteinase K on lipid mixing was monitored by incubating untreated liposomes with RBC ghosts in presence of equivalent amount of proteinase K.

Figure 14 Dextran loaded DOTAP/*N*-Ac-AA-DOPE/PE liposome fusion with RBC ghosts. DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes were loaded with 10kD TX-red conjugated dextrans. Liposomes were incubated with proteinase K overnight at 37°C. 40nmol aliquots of dextran loaded liposomes or unloaded liposomes + free dextran were incubated with $1x10^8$ RBC ghosts in 1 ml buffer for 30min at 37°C, after which cells were washed and observed by fluorescence microscopy or Nomarski differential interference contrastmicroscopy (Fig. 14A depicts results with dextran-loaded liposomes, using fluorescence microscopy; Fig. 14B depicts results with unloaded liposomes, using fluorescence microscopy; Fig. 14C. depicts results with dextran-loaded liposomes, using Nomarski contrast microscopy; Fig. 14D. depicts results with unloaded liposomes, using Nomarski contrast microscopy).

Figure 15 Optimum DODAP/MeO-suc-AAPV-DOPE liposome composition for elastase activation of binding/lipid mixing with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes prepared at 1:3, 1:1, or 3:1 (mol/mol) ratios and labeled with 0.75 mol% N-NBD-PE and 0.75 mol% N-Rho-PE were incubated with or without elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. 10nmol of liposomes were then mixed with 1x106 HL60 cells and incubated for 30min at 37oC, pH 5. After washing twice with 5x volume of TES/NaCl/EDTA buffer, pH 7.4, A) binding and B) lipid mixing was determined by monitoring N-Rho-PE and N-NBD-PE fluorescence, respectively.

Figure 16. pH dependence of DODAP/MeO-suc-AAPV-DOPE liposome binding/lipid mixing with HL60 cells. Fluorescent lipid probe labeled DODAP/MeO-suc-AAPV-DOPE liposomes were incubated with or without elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. 10nmol of liposomes were mixed with 1x106 HL60 cells in 200 • l TES/NaCl/EDTA buffer. Samples were incubated for 30min, 37oC, at the given pH and washed. Liposome binding (Fig. 16A) and lipid mixing (Fig. 16B) were determined by monitoring N-Rho-PE and N-NBD-PE fluorescence, respectively.

Figure 17 Active elastase is required for triggering DODAP/MeO-suc-AAPV-DOPE liposome binding/lipid mixing with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes containing 0.75 mol% N-NBD-PE and 0.75 mol% N-Rho-PE were incubated

alone, with elastase (5 • g elastase/100 nmol lipid), or with equivalent amount of heat inactivated elastase (95oC, 1 hour) for 2 hours at 37oC. 10nmol of pretreated liposomes were mixed with 1x106 HL60 cells in 200 • l TES/NaCl/EDTA buffer. Samples were incubated for 30min, 37oC, at either pH 7.4 or pH 5 and washed. Liposome binding (Fig. 17A) and lipid mixing (Fig. 17B) was determined as previously described.

Figure 18 Confocal microscopy of DODAP/MeO-suc-AAPV-DOPE liposome interaction with HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes labeled with 0.75 mol% N-NBD-PE and 0.75 mol% N-Rho-PE were incubated alone or with elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC as described in Fig. 17. After washing, cells were observed for N-Rho-PE fluorescence. Color range employed as in Figure 19.

Figure 19 Delivery of TMR-dextran from DODAP/MeO-suc-AAPV-DOPE liposomes to HL60 cells. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with 10,000 MW TMR-dextran were incubated alone or with elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. Liposomes were then added directly to cells or subjected to 5 freeze/thaw cycles to release encapsulated TMR-dextran. 40 nmol of intact or freeze/thawed liposomes were mixed with 2x105 HL60 cells and incubated for 30 min at 37oC, pH 5. After washing, cells were observed for TMR-dextran fluorescence by confocal microscopy. Color range of 0-255 pixel values (0, bottom; 255, top) are given in upper right corner of each image.

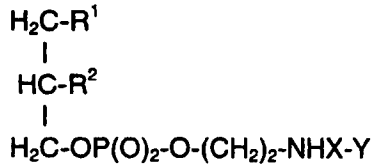
Figure 20 Binding/lipid mixing of DODAP/MeO-suc-AAPV-DOPE liposomes with ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes containing N-NBD-PE and N-Rho-PE were incubated alone or with elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. 100nmol of pretreated liposomes were added to ECV304 cells that had been pretreated with biotinylated wheat germ agglutinin (WGA) and streptavidin as described in the Examples). After a 30min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for additional 2 hours at 37oC. Binding and lipid mixing was determined by monitoring N-Rho-PE and N-NBD-PE fluorescence, respectively.

Figure 21 Confocal microscopy of elastase-activated calcein delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with quenched concentration of calcein were incubated alone or with elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. 100nmol of pretreated intact liposomes were added to ECV304 cells that had been pretreated with biotinylated WGA and streptavidin After a 30 min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for an additional 4 hours at 37oC. Cells were observed by confocal microscopy for calcein fluorescence. Color range of 0-255 pixel values (0, bottom; 255, top) are given in upper right corner of each image.

Figure 22 Elastase-triggered calcein delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to ECV304 cells at pH 7.4. DODAP/MeO-suc-AAPV-DOPE liposomes loaded with self-quenched concentration of calcein and N-Rho-PE as a lipid marker were incubated with or without elastase (5 • g elastase/100 nmol lipid) for 2 hours at 37oC. 100nmol liposomes were added to ECV304 cells that had been pretreated with biotinylated WGA and streptavidin. After a 30min incubation at room temperature the cell monolayer was washed to remove unbound liposomes and samples were incubated for 0, 1, 2, 4, or 6 hours at 37oC. At given times calcein delivery was determined by monitoring calcein fluorescence.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a peptide-lipid conjugate having the following formula:



wherein: each of R^1 and R^2 is independently a group having the formula $-OC(O)(CH_2)_{n1}(CH=CH)_{n2}(CH_2)_{n3}(CH=CH)_{n4}(CH_2)_{n5}(CH=CH)_{n6}(CH_2)_{n7}$

$(CH=CH)_{n8}(CH_2)_{n9}CH_3$ and X is a linker moiety selected from the group consisting of a single bond and an acyl chain having the formula:
$-C(O)(CH_2)_{n1}(CH=CH)_{n2}(CH_2)_{n3}(CH=CH)_{n4}(CH_2)_{n5}(CH=CH)_{n6}(CH_2)_{n7}(CH=CH)_{n8}(CH_2)_{n9}HN-$.

n1 is equal to zero or an integer of from 1 to 22, n3 is equal to zero or an integer of from 1 to 19, n5 is equal to zero or an integer of from 1 to 16, n7 is equal to zero or an integer of from 1 to 13 and n9 is equal to zero or an integer of from 1 to 10; and, each of n2, n4, n6 and 8 is independently zero or 1. For R^1 and R^2, the sum of n1 + 2n2 + n3 + 2n4 + n5 + 2n6 + n7 + 2n8 + n9 is equal to an integer of from 10 to 22.

X is preferably a single bond; however, when X is other than a single bond, the sum of n1 + 2n2 + n3 + 2n4 + n5 + 2n6 + n7 + 2n8 + n9 for X is equal to an integer of from 1 to 22. X is then preferably saturated, most preferably:
$-C(O)(CH_2)_{11}HN-$.

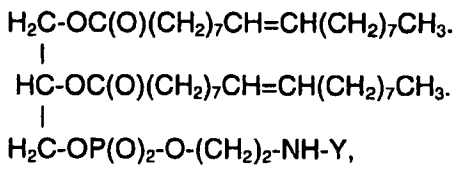
Preferably, at least one of R^1 and R^2 contains at least one double bond, and the peptide-lipid conjugate is thus partially or completely unsaturated. More preferably, both of R^1 and R^2 contain one double bond, and the conjugate is thus completely unsaturated. Most preferably, presently, both R^1 and R^2 are $-OC(O)(CH_2)_7(CH=CH)(CH_2)_7CH_3$, i.e., the peptide-lipid conjugate is a dioleoyl phosphatidylethanolamine (DOPE)-based conjugate. However, each of R^1 and R^2 can also be saturated or unsaturated acyl chains that include, without limitation: $-OC(O)(CH_2)_{14}CH_3$, $-OC(O)(CH_2)_{16}CH_3$, $-OC(O)(CH_2)_{18}CH_3$ or $-OC(O)(CH_2)_8(CH=CH)(CH_2)_8CH_3$.

Y is an "enzyme-cleavable peptide," which is a peptide comprising an amino acid sequence that is recognized by a peptidase expressed by a mammalian cell and found in surrounding tissue, or produced by a microbe capable of establishing an infection in a mammal. Enzyme-cleavable peptides can, but are not required to, contain one or more amino acids in addition to the amino acid recognition sequence; additional amino acids

or other groups (such as methoxysuccinyl, PEG, acetyl) can be added to the amino terminal, carboxy terminal, or both the amino and carboxy terminal ends of the recognition sequence. Means of adding amino acids to an amino acid sequence, e.g., in an automated peptide synthesizer, as well as means of detecting cleavage of a peptide by a peptidase, e.g., by chromatographic analysis for the amino acid products of such cleavage, are well known to ordinarily skilled artisans given the teachings of this invention.

Enzyme-cleavable peptides, typically from about 2 to 20 amino acids in length, are of sufficient length to project above the surfaces of lipid-based carriers into which they have been incorporated. Such peptides are well known to ordinarily skilled artisans given the teachings of this invention and include, for example and without limitation, the amino acid sequences: Ala-Ala-, Ala-Ala-Pro-Val , Ala-Ala-Met-, Ala-Ala-Pro-Phe-, Ala-Ala-Pro-Met-, Ala-Ala-Arg, Ser-Ala-Ala-Arg-, Ser-Ser-Ala-Ala-Arg-, Ser-S carboxyl sugar-Ala-Ala-Arg-, Ala-Ala-Asp-, Ser-Ala-Ala-Asp-, Ser-Ser-Ala-Ala-Asp-, Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ser-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva, Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2, Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Pro-Cha-Gly-Nva-, Pro-Leu-Gly-Leu, Gly-Pro-Arg, Leu-Pro-Arg, Glu-Gly-Arg, Pro-Leu-Gly-Leu- and Gly-Pro-Gln-Gly-Ile-. Presently, the preferred peptides comprise the amino acid sequence Ala-Ala, more preferably, Ala-Ala-Pro-Val; and most preferably, N-methoxysuccinyl-Ala-Ala-Pro-Val.

Accordingly, the peptide-lipid conjugate of this invention most preferably has the formula:



wherein the peptide comprises the amino acid sequence N-methoxysuccinyl-Ala-Ala-Pro-Val.

Enzyme-cleavable peptides can be modified at their amino terminii, for example, so as to increase their hydrophilicity, the stability of the liposome, or to enhance enzyme activity. Increased hydrophilicity enhances exposure of the peptides on the surfaces of lipid-based carriers into which the parent peptide-lipid conjugates have been incorporated and can increase the liposome's stability. Groups suitable for attachment to peptides so as to increase their hydrophilicity are well known, and include, for example and without limitation: acetyl ("Ac"), acetyl-serine ("Ac-Ser"), acetyl-seryl-serine ("Ac-Ser-Ser-"), succinyl ("Suc"), succinyl-serine ("Suc-Ser"), succinyl-seryl-serine ("Suc-Ser-Ser"), methoxy succinyl ("MeO-Suc"), methoxy succinyl-serine ("MeO-Suc-Ser"), methoxy succinyl-seryl-serine ("MeO-Suc-Ser-Ser") and seryl-serine ("Ser-Ser-") groups, polyethylene glycol ("PEG"), polyacrylamide, polyacrylomorpholine, polyvinylpyrrolidine, a polyhydroxyl group and carboxy sugars, e.g., lactobionic, N-acetyl neuraminic and sialic acids, groups. The carboxy groups of these sugars would be linked to the N-terminus of the peptide via an amide linkage. Specific groups, such as acetyl, succinyl, methoxysuccinyl or 3-cyclohexylalanyl ("Cha") groups, although not hydrophilic, enhance enzyme activity. Specific groups, such as polyethylene glycol , polyacrylamide, polyacrylomorpholine, polyvinylpyrrolidine, a polyhydroxyl group and carboxy sugars, e.g., lactobionic, N-acetylneuraminic and sialic acids, may additionally block exposure of the lipid bilayer to serum or other cellular components, priot to cleavage at the peptide-linkage by a peptidase. Presently, the preferred N-terminal modification is an acetyl modification, and the more preferred modification is a methoxy-succinyl modification.

For purposes of the present invention, the term extracellular enzyme is defined to include enzymes that are produced by cells and that are released into the medium or other areas surrounding the cell and is free from the cell; it is also meant to include enzymes which are produced by cells and secreted outside the cell, and which may remain in the immediate area of the cell, and also includes enzymes which are secreted by cells, which remain either bound to or associated with, the cell's outer surface. This definition is also meant to encompass the cell-secreted peptidases described below, and enzymes produced by mammalian cells or by microbes.

Cell-secreted peptidases which recognize particular amino acid sequences are also well known to ordinarily skilled artisans given the teachings of this invention. Such peptidases include, for example and without limitation: matrix metalloproteinases, serine proteases, cysteine proteases, elastase, plasmin, plasminogen activators, such as tissue plasminogen activator and urokinase, stromelysin, human collagenases, cathepsins, lysozyme, granzymes, dipeptidyl peptidases, peptide hormone-inactivating enzymes, kininases, bacterial peptidases and viral proteases. Elastase, for example, is involved in tumor cell tissue remodeling; the breast cancer cell line MCF-7 has been shown to secrete elastase, the levels of which are inversely correlated to overall survival in breast cancer patients (Yamashita et al.). Moreover, the matrix metalloproteinase, stromelysin-3 ("ST3"), has been localized to the stromal area of tumor cells (Pei et al, 1994.); it specifically cleaves α_1 proteinase inhibitor between amino acids 350 and 351 (Ala-Met). Stromelysin-1 ("MMP-3") is also localized to areas of tissue remodeling, including sites of inflammation and tumor stroma (Nagase et al., 1994). Metastatic cancer cells display enhanced extracellular activity of, among others, the matrix metalloproteinases and urokinase-type plasminogen activator (reviewed in Liotta et al., 1991).

The cDNA of human collagenase-3 or MMP-13, another metalloproteinase was isolated from a breast tumor library (Knäuper et al., 1996); this enzyme cleaves peptides containing the amino acid sequences Pro-Cha-Gly-Nva-His- and Pro-Leu-Gly-Leu-. Furthermore, the 72 kDa gelatinase (MMP-2) is involved in regulating tumor cell invasiveness, and cleaves the amino acid sequence Gly-Pro-Gln-Gly-Ile- between the Gly and Ile residues (Aimes and Quigley, 1995; Liotta et al., 1991). Human neutrophils also secrete collagenases at sites of inflammation such as MMP-8 (neutrophil collagenase) and MMP-9 (type IV collagenase, 92 kDa gelatinase) (Fosang et al., 1994). Cathepsin G is also secreted from human neutrophils at sites of inflammation; its specificity is greatest for peptides containing the amino acid sequences Suc-Ala-Ala-Pro-Phe- or MeOSuc-Ala-Ala-Pro-Met- (Nakajima et al., 1979). Other enzymes secreted by neutrophils at sites of inflammation include cathepsins B and D as well as lysozyme. Granzymes A and B are secreted by cytotoxic lymphocytes in the synovial fluid of rheumatoid arthritis patients (Froehlich et al., 1993); granzyme A cleaves

peptides comprising Gly-Arg- and Ala-Ala-Arg- most efficiently, while granzyme B cleaves peptides comprising the amino acid sequence Ala-Ala-Asp (Odake et al., 1991).

Peptidases which hydrolyze enzyme-cleavable peptides also include the group of enzymes that inactivate peptide hormones, e.g., aminopeptidase P and angiotensin-converting enzyme, localized on the surface of endothelial cells. Aminopeptidase P cleaves the Arg-Pro bond in bradykinin, and is localized to lung endothelial cells (Prechel et al., 1995).

Numerous pathological conditions, some of which have already been discussed, are associated with elevated enzyme activity. Metastatic cancer cells display enhanced extracellular activity of several degradative enzymes, [for review see Liotta et al., 1991]. Elevated enzymatic activity facilitates the extravasation of these cells from the circulation and increases their invasive potential. Other disorders, such as the inflammatory conditions cystic fibrosis [McElvaney et al, 1991; Rees and Brain, 1995; Berger et al, 1989, and Suter et al., 1986], rheumatoid arthritis [Al-haik et al, 1984, and Gysen et al, 1985], and emphysema [Damiano et al, 1986, and Snider et al., 1991] are accompanied by an increase in extracellular elastase activity due to release of elastase from phagocytic cells. Elevated elastase activity appears to be due, in part, to an imbalance in the elastase/anti-protease ratio [McElvaney et al., 1991, Cavarra et al., 1996, and Doring et al., 1994]. Elastase has also been associated with tumor progression and development [Yamashita et al., 1994, Yamashita et al., 1997, and Starcher et al., 1996]. The ubiquitous yet specific nature of disease-associated enzymatic activity, its localization near or on the membranes of cells involved in tissue remodeling [Sato et al., 1994 and Owen et al., 1995] and its association with several pathologies provide numerous opportunities for triggering specific liposomal delivery to desired targets using the activity of such enzymes. The triggering event would be expected to convert the liposome from a relatively inert state to a fusogenic state and may even trigger specific binding depending on the design.

The selectivity of liposomal activation can be modulated by the choice of an enzyme substrate conjugated to a fusogenic lipid such that enzymatic cleavage releases

or unmasks fusogenic lipids. Thus liposomes may be designed for a selected site of activation and hence liposomal delivery could be targeted.

Peptide-lipid conjugates are prepared by any of a number of means for forming an amide bond between the amino group of a phosphatidylethanolamine and the carboxy terminus of an amino acid sequence. Such means include, without limitation, those described in Example 1, hereinbelow. Briefly, an enzyme-cleavable peptide containing an N-terminal blocking group is prepared as an anhydride; a phosphatidylethanolamine such as DOPE is then reacted with the anhydride in the presence of suitable reagents, such as triethylamine.

This invention also provides a liposome having a lipid component which comprises the peptide-lipid conjugate of the invention. "Liposomes" are self-assembling structures comprising one or more lipid bilayers, each of which surrounds an aqueous compartment and comprises two opposing monolayers of amphipathic lipid molecules. Amphipathic lipids comprise a polar (hydrophilic) headgroup region covalently linked to one or two non-polar (hydrophobic) acyl chains. Energetically unfavorable contacts between the hydrophobic acyl chains and the aqueous medium are generally believed to induce lipid molecules to rearrange such that the polar headgroups are oriented towards the aqueous medium while the acyl chains reorient towards the interior of the bilayer. An energetically stable structure is formed in which the acyl chains are effectively shielded from coming into contact with the aqueous medium.

Liposomes of this invention can have a single lipid bilayer (unilamellar liposomes, "ULVs"), or multiple lipid bilayers (multilamellar liposomes, "MLVs"), and can be made by a variety of methods well known in the art. These methods include without limitation: Bangham's methods for making multilamellar liposomes (MLVs); Lenk's, Fountain's and Cullis' methods for making MLVs with substantially equal interlamellar solute distribution (see, for example, U.S. Patent Nos. 4,522,803, 4,588,578, 5,030,453, 5,169,637 and 4,975,282); and Papahadjopoulos et al.'s reverse-phase evaporation method (U.S. Patent No. 4,235,871) for preparing oligolamellar liposomes. ULVs can

be produced from MLVs by such methods as sonication or extrusion (U.S. Patent No. 5,008,050 and U.S. Patent No. 5,059,421). The liposome of this invention can be produced by the methods of any of these disclosures, the contents of which are incorporated herein by reference.

Various methodologies, such as sonication, homogenization, French Press application and milling can be used to prepare liposomes of a smaller size from larger liposomes. Extrusion (see U.S. Patent No. 5,008,050) can be used to size reduce liposomes, that is to produce liposomes having a predetermined mean size by forcing the liposomes, under pressure, through filter pores of a defined, selected size. Tangential flow filtration (see WO89/008846), can also be used to regularize the size of liposomes, that is, to produce a population of liposomes having less size heterogeneity, and a more homogeneous, defined size distribution. The contents of these documents are incorporated herein by reference. Liposome sizes can also be determined by a number of techniques, such as quasi-elastic light scattering, and with equipment, e.g., Nicomp® particle sizers, well within the possession of ordinarily skilled artisans.

Liposomes of this invention can have lipid components entirely composed of a peptide-lipid conjugate. However, the liposomes preferably contain one or more additional lipids, including any of those lipids, such as phospholipids, glycolipids and sterols, typically used to prepare liposomes. Preferably, the additional lipid is a positively charged lipid, more preferably such a lipid selected from the group consisting of 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP), 1-N,N-dimethylamino dioleoyl propane (DODAP), 1-oleoyl- 2-hydroxy-3-N,N-dimethylamino propane, 1,2-diacyl-3-N,N-dimethylamino propane and 1,2-didecanoyl -1-N,N,-dimethylamino propane, 3β-[N-[(N',N'-dimethylamino)ethane]carbamoyl]cholesterol (DC-Chol), 1,2-dimyristooxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE) and 1,2-dioleooxypropyl-3-dimethylhydroxyethyl ammonium bromide (DORI).

Most preferably, presently, the positively charged lipid is DODAP. Positively charged lipids are incorporated into the liposomes, preferably in at most about equimolar concentration respective to the peptide-lipid conjugate, in order to adjust the

net charge of the carrier. Increasing the positive charge on a lipid-based carrier enhances electrostatic interactions between the carrier and a biological membrane and hence, fusion between the carrier and the membrane.

The additional lipid can also include one or more phospholipids, such as a phosphatidylcholine ("PC"), which are generally added to lipid carriers to serve as structural stabilizers, or a phosphatidylethanolamine ("PE"). The PE may be selected from the group consisting of trans-esterified phosphatidylethanolamine (tPE), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE) and dioleolyl phosphatidylethanolamine (DOPE); such additional PE's can be fusogenic because of the relatively unhydrated state of their headgroups.

Alternatively, the PE is a PE to the headgroup of which is attached a moiety selected from the group consisting of dicarboxylic acids, polyethylene glycols, polyalkyl ethers and gangliosides. Such modified PEs, also known as "headgroup-modified lipids," can inhibit the binding of serum proteins to lipid carriers such that the pharmacokinetic behavior of the carriers in the circulatory systems of animals is altered (see, e.g., Blume et al., 1993, Gabizon et al., 1993; Park et al., 1992, Woodle et al., and Allen et al.; the contents of which are incorporated herein by reference). The amount of the headgroup-modified lipid incorporated into the liposomes depends upon a number of factors well known to the ordinarily skilled artisan, or within his purview to determine without undue experimentation. These include, but are not limited to: the type of lipid and the type of headgroup modification; the type and size of the liposome; and the intended therapeutic use of the formulation. The concentration of the headgroup-modified lipid in the liposome is generally sufficient to prolong the liposome's circulatory half-life in an animal, but is not so great as induce unwanted side effects in the animal, and is typically at least about five mole percent of the lipid present in the liposome. Preferred headgroup-derivatized lipids include phospatidylethanolamine-dicarboxylic acids ("PE-DCAs") and polyethyleneglycol- modified (PEGylated) lipids (for a description of which, see Woodle et al. and Allen et al.).

The liposome of this invention can comprise a "targeting moiety," i.e., a moiety that can be attached to a liposome and which can then direct the liposome to a specific site within the body of a mammal. Such directed delivery is generally believed to occur as a result of the recognition by the targeting moiety of a compound on the surface of the cells being targeted. Typical targeting moieties include, without limitation, antibodies, cell receptor ligands, lectins and the like. Targeting moieties can be attached to liposomes by any of the means generally accepted in the art for the covalent or noncovalent attachment of such moieties to liposomes. Such means include, for example and without limitation, those described in the following documents, the contents of which are incorporated herein by reference: U.S. Patent No. 5,399,331 describes the coupling of proteins to liposomes through use of a crosslinking agent having at least one maleimido group and an amine reactive function; U.S. Patent Nos. 4,885,172, 5,059,421 and 5,171,578 link proteins to liposomes through use of the glycoprotein streptavidin; Sato and Sunamoto (1993) describe the coating of targeted liposomes with polysaccharides.

The liposomes of this invention can comprise one or more "bioactive agents," which are compounds or compositions of matter having biological, including therapeutic or diagnostic, activity in animals. Bioactive agents which may be associated with the liposomes include, but are not limited to: antiviral agents such as acyclovir, zidovudine and the interferons; antibacterial agents such as aminoglycosides, cephalosporins and tetracyclines; antifungal agents such as polyene antibiotics, imidazoles and triazoles; antimetabolic agents such as folic acid analogues, folic acid antagonists including methotrexate, and purine and pyrimidine analogs; antineoplastic agents such as the anthracycline antibiotics and plant alkaloids; sterols such as cholesterol; carbohydrates, e.g., sugars and starches; amino acids, peptides, proteins such as cell receptor proteins, immunoglobulins, enzymes, hormones, neurotransmitters and glycoproteins; dyes; radiolabels such as radioisotopes and radioisotope-labeled compounds; radiopaque compounds; fluorescent compounds; mydriatic compounds; bronchodilators; local anesthetics; nucleic acid sequences such as messenger RNA, cDNA, genomic DNA and plasmids; bioactive lipids such as ether lipids and ceramides; and the like.

Preferred bioactive agents are selected from the group consisting of nucleic acid sequences, antimicrobial agents, anticancer agents and anti-inflammatory agents.

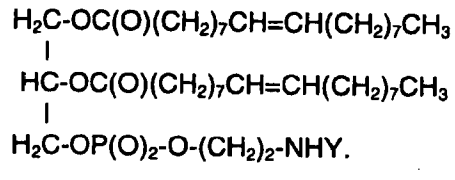
A "blocking group" as used herein refers to a lipid component of the liposome or cell membrane which sterically or physically blocks a fusogenic component of the membrane from being expressed. Thus, removal of a portion or all of a blocking group by enzymatic activity may also render the liposome or cell membrane fusogenic.

The term "effector" as used in this specification is defined as a compound or liposome which is added to other liposomes or cells for the purpose of fusing with the other liposome or cell, defined as the "acceptor". The effector liposome thus can be used to deliver a bioactive agent to a cell.

An endocytic vesicle (or endocytic compartment) is a membrane-bound vesicle that is formed by endocytosis and contains extracellular materials that are to be delivered to any of several destinations within the cell.

An endosome is the cytoplasmic vesicle formed from endocytic vesicles that have shed their clathrin coats, or formed directly from coated pits. The pH of endosomes and endocytic vesicles is acidic.

Preferably, the liposome has a lipid component which comprises a positively charged lipid and a peptide-lipid conjugate having the formula:



More preferably, the peptide comprises the sequence N-methoxy-succinyl-Ala-Ala-Pro-Val and the positively charged lipid is DODAP. Most preferably, presently, the lipid component comprises DODAP and the peptide-lipid conjugate in a respective molar ratio of about 50:50.

Further provided herein is a composition comprising the liposome and a "pharmaceutically acceptable carrier," which is a medium generally acceptable for use in connection with the administration of liposomes to mammals, including humans. Pharmaceutically acceptable carriers are formulated according to a number of factors well within the purview of the ordinarily skilled artisan to determine and account for, including without limitation: the particular liposomal bioactive agent used, its concentration, stability and intended bioavailability; the disease, disorder or condition being treated with the liposomal composition; the subject, its age, size and general condition; and the composition's intended route of administration, e.g., nasal, oral, ophthalmic, topical, transdermal, vaginal, subcutaneous, intramammary, intraperitoneal, intravenous, or intramuscular (see, for example, Nairn (1985)). Typical pharmaceutically acceptable carriers used in parenteral bioactive agent administration include, for example, D5W, an aqueous solution containing 5% weight by volume of dextrose, and physiological saline. Pharmaceutically acceptable carriers can contain additional ingredients, for example those which enhance the stability of the active ingredients included, such as preservatives and anti-oxidants.

Still further provided is a method of delivering the contents of a liposome to a cell which comprises contacting the cell with the liposome of this invention in the presence of a protease capable of cleaving the peptide-lipid conjugate. Delivery can occur *in vitro*, such as for diagnostic purposes or for *ex vivo* delivery of a therapeutic agent or nucleic acid to bone marrow cells. *In vitro* contact of a biological membrane with a lipid-based carrier involves adding the carrier-containing composition of this invention to cultures of protease-secreting cells, including various tumor cell lines such as the MCF-7 line, or adding an endogenous protease to the culture medium containing the membranes and the carriers.

Still further provided is a method of delivering the contents of a liposome to nucleated mammalian cells, where at least some of the delivery may occur via an endosomal compartment of the cell. In this method liposomes were developed to take advantage of the low pH of the endosomal compartment in a manner that would

enhance enzyme (elastase) triggering. This is the first report of a liposomal system which can be triggered by physiological levels of elastase to undergo lipid mixing with, and delivery of the liposomes' aqueous contents to nucleated cells. As will be described further in the examples which follow, these activities can occur in two types of human cell lines, a leukemia cell line (HL60) and an adherent cell line, ECV304.

Alternatively, the contacting can be *in vivo*, in which case the cells are preferably mammalian, a pharmaceutically acceptable carrier is used and the liposomes preferably comprise a targeting moiety. *In vivo* administration involves administering the compositions of this invention to the mammal by any of the means, e.g., by intravenous administration, generally accepted in the art for administering pharmaceutical compositions to mammals. The carriers will then circulate in the mammals, and will become fusogenic in the presence of peptidase concentrations sufficient to cleave the carriers' peptide-lipid conjugates; as described hereinabove, such peptidases are found in mammals at, for example, sites of inflammation, microbial infection and tumors. Moreover, incorporation of headgroup-modified lipids into lipid-based carriers increases the amount of time the carriers remain in circulation, and hence the proportion of the administered carrier reaching the intended site of action within the mammal. Furthermore, tumors generally have a higher degree of vasculature than does surrounding tissue, and these blood vessels are typically more permeable to structures such as lipid-based carriers. Accordingly, the carriers accumulate in tumors, thus further enhancing the proportion of administered carrier reaching the intended site of therapeutic action. Fusion in vivo can be to the cells secreting the protease as well as to nearby cells in the surrounding tissue.

In vivo liposomal bioactive agent delivery according to the practice of this invention can deliver therapeutically or diagnostically effective amounts of therapeutic or diagnostic agents into the cells of a mammal afflicted with a disease, disorder or condition amenable to diagnosis or treatment with the agent. Hence, such delivery can be used to diagnose or treat the mammal for the disease, disorder or condition.

The method of this invention can also be used to treat mammals afflicted with inflammatory disorders, by administering to the mammal a liposome containing an anti-inflammation effective amount of an anti-inflammatory agent. Treatable inflammatory disorders include, without limitation, arthritic disorders, autoimmune disorders, atherosclerotic plaque, acute respiratory distress syndrome, inflammatory bowel syndrome, acute nephritis or gout; suitable anti-inflammatory agents include, without limitation, nonsteroidal anti-inflammatory agents, glucocorticoids, bioactive lipids such as ceramides and ether lipids, and prostaglandins. Peptidases known to be present at sites of inflammation include, without limitation: elastase, which recognizes Ala-Ala- and cleaves peptides such as Ala-Ala-, Ala-Ala-Ala-, Ala-Ala-Pro-Val, Ala-Ala-Pro-Met and Ala-Ala-Pro-Ala; stromelysin-1, which recognizes peptides comprising the amino acid sequence Arg-Pro-Lys-Pro-Leu-Ala-Nva-, such as Ac-Arg-Pro-Lys-Pro-Leu-Ala-Nva, MeOSucArg-Pro-Lys-Pro-Leu-Ala-Nva-, carboxy sugar-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Suc-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ac-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, MeOSuc-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ser-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva-, Ac-Ser-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva- and MeOSuc-Ser-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva, and which cleaves the peptides at the Ala-Nva bond; and, cathepsin G, which is secreted by human neutrophils secreted at the site of inflammation, and cleaves peptides such as Suc-Ala-Ala-Pro-Phe-, carboxy sugar-Ala-Ala-Pro-Phe-, MeOSuc-Ala-Ala-Pro-Met-, Suc-Ala-Ala-Pro-Met, and carboxy sugar-Ala-Ala-Pro-Met-.

Moreover, peptide substrates for the enzymes granzyme A and granzyme B, secreted by cytotoxic lymphocytes in the synovial fluid of rheumatoid arthritis patients, include, without limitation: Ac-Ala-Ala-Arg-, MeOSuc-Ala-Ala-Arg-, Ala-Ala-Arg-, Ser-Ala-Ala-Arg-, Ac-Ser-Ala-Ala-Arg-, MeOSuc-Ser-Ala-Ala-Arg-, Ser-Ser-Ala-Ala-Arg-, Ac-Ser-Ser-Ala-Ala-Arg-, MeOSuc-Ser-Ser-Ala-Ala-Arg- and carboxyl sugar-Ala-Ala-Arg-, etc. Ac-Ala-Ala-Asp-, MeOSuc-Ala-Ala-Asp-, Ala-Ala-Asp-, Ser-Ala-Ala-Asp-, Ac-Ser-Ala-Ala-Asp-, MeOSuc-Ser-Ala-Ala-Asp-, Ser-Ser-Ala-Ala-Asp-, Ac-Ser-Ser-Ala-Ala-Asp-, MeOSuc-Ser-Ser-Ala-Ala-Asp-, and carboxyl sugar-Ala-Ala-Asp-. Dipeptidylaminopeptidase IV (DAP IV, EC 3.4.14.5), a member of the dipeptidyl peptidase enzyme family, is found in increased concentrations on pig aorta smooth

muscle cells (Palmieri et al., 1989). Vessel wall damage, e.g., after angioplasty or during other inflammatory states exposes the peptidase. For instance, inflammatory edema is associated with breach of the endothelial lining and exposure of smooth muscle cells. Appropriate substrates could be used for liposomal delivery to these sites.

The method of this invention can also be used to treat mammals afflicted with cancers, by administering to the mammals a liposome containing an anticancer effective amount of an anticancer agent. Treatable cancers include brain, breast, colon, lung, ovarian, prostate and stomach cancers, as well as sarcomas, carcinomas, leukemias, lymphomas and melanomas; suitable anticancer agents include, without limitation, anthracycline antibiotics, bioactive lipids such as ceramides and ether lipids, taxanes and vinca alkaloids. Peptidases known to be present in the vicinity of tumors include, for example and without limitation: elastase, which cleaves peptides containing the amino acid sequence Ala-Ala-, Ala-Ala-Pro-Val (Nakajima et al.,1979, Castillo et al., 1979); stromelysin-3, which cleaves peptides containing the amino acid sequence Ala-Met; stromelysin-1, which cleaves peptides containing the amino acid sequence Ala-Nva-; human collagenase-3, which cleaves peptides such as MeOSuc-Pro-Cha-Gly-Nva-, Suc-Pro-Cha-Gly-Nva-, Pro-Cha-Gly-Nva-, Pro-Leu-Gly-Leu-, MeOSuc-Pro-Leu-Gly-Leu- and Suc-Pro-Leu-Gly-Leu-; and, the 72-kD gelatinase, which cleaves peptides containing the amino acid sequence Gly-Pro-Gln-Gly-Ile- (see Pei et al., 1994; Knäuper et al., 1996; Boyd, 1996; Unden et al., 1996; and, Kossakowska et al, 1996.) and urokinase plasminogen activator, which cleaves Glu-Gly-Arg and Ac-Lys (Wohl et al., 1980; Johnson et al., 1969; Petkov et al., 1975; Ascenzi et al., 1980), and cathepsin B, which cleaves Arg-Arg (Knight, 1980; Barrett & Kirschke, 1981; Kirschke et al., 1982).

Moreover, specific peptidases are also found in neuronal tissue (e.g. O'Leary and O'Connor, 1995), suggesting that the liposomes may be designed to treat several neuropathies. Specific aminopeptidases are produced on the membranes of the placental tissue and later secreted suggesting primary localization of this activity in the placenta (Rogi et al., 1996). Several kininases are localized to the kidney. For example renin is found in the zona glomerulosa and/or adrenal medulla (Berka et al., 1996). Certain peptidases have even been identified in skeletal muscle (Ward et al., 1995).

Observation of strong activity of an alanylaminopeptidase in the stroma of basal cell carcinoma and DAP IV in the tumor cells themselves (Moehrle et al., 1995) suggest an alanyl-phospholipid or appropriate dipeptides as possible triggers for liposomal fusion with tumor cells.

One of ordinary skill in the art, having chosen a protease from the enzymes described above, or those specifically employed in the Examples described below, would know the cleavage site where cleavage is occurring, and would readily know where the cleavage site is on a particular peptide without undue experimentation.

The method of this invention can also be used to treat mammals afflicted with microbial infections, by administering to the mammals a liposome containing an anti-infection effective amount of an anti-infective agent, such as the various antibiotics. A number of specific peptidases are associated with certain bacteria and may be utilized to deliver liposomal contents to sites of infection (e.g. Spratt et al., 1995). Human immunodeficiency viruses have proteases with particular specificities (e.g. Hoog et al.) that may be expressed in or near infected cells and may be utilized to target fusogenic liposomes for therapy.

The contents of the above-cited documents, with their descriptions of secreted enzymes and their target peptides, are incorporated herein by reference.

Liposomal drug delivery according to the practice of this invention can direct the liposomes contents to the vicinity of the target cells. It can also deliver the contents directly into cells, by way of fusion between the liposomes and the cells. "Fusion" of a liposome to a cell involves both binding of the liposome to the cell, as well as mixing of liposomal and cell membrane lipids. Binding and lipid mixing can be assessed by a number of means well known to ordinarily skilled artisans given the teachings of this invention including, for example, those described hereinbelow.

Briefly, liposomes are labeled by incorporation therein of a fluorescent marker and mixed with erythrocyte ghosts, prepared as described hereinbelow. Erythrocyte ghosts are incapable of endocytosis, and hence, any transference of fluorescence between the liposome and ghosts must be due to fusion of the liposome with the plasma membrane. Measurement of erythrocyte ghost fluorescence is thus a measure of the fusion of liposome to the ghosts. Fusion may also occur within the endosome of a living nucleated cell between the liposomal and endosomal membranes, so as to deliver the liposomes' encapsulated material into the cell. Peptidase-mediated cleavage of a peptide-lipid conjugate herein converts a nonfusogenic liposome into a fusogenic liposome. Moreover, the liposome can contain one or more additional fusogenic lipids, including PE's such as DOPE and synthetic lipids such as DOTAP and DODAP. Such lipids promote fusion of their parent liposomes to adjacent lipidic membranes, because of the nonbilayer structures adopted by the lipids in aqueous environments.

However, the peptide-lipid conjugate can also contain a "blocking" group, e.g., a carboxy sugar such as lactobionic acid or N-acetyl neuraminic acid, or a polymeric compound such as a small polyethylene glycol derivative, a polyhydroxyl polymer or a number of other amino acids of a composition containing hydrophilic side chains such as serine or threonine. This blocking group is attached to the N-terminus of the peptide, and inhibits or blocks the liposome and lipidic membrane from approaching closely enough for fusion between the two to occur. Cleavage of the peptide by a protease removes this N-terminal blocking group from the peptide, and hence, allows for fusion between the liposome and the lipidic membrane. Peptidase-mediated cleavage thus, by cleaving the peptide portion of the peptide-lipid conjugate, results in the generation of a fusogenic liposome.

This invention will be better understood in light of the following Examples. However, those of ordinary skill in the art will readily understand that the examples are merely illustrative of the invention as defined in the claims which follow thereafter.

EXAMPLES

Example 1

A) Chemical Synthesis of N-Ac-Ala-Ala-DOPE

N-acetyl-alanyl-alanyl-dioleoyl phosphatidylethanolamine ("N-Ac-Ala-Ala-DOPE") was synthesized by first preparing an anhydride form of the peptide from N-acetyl-ala-ala-OH, or other suitably blocked carboxyl-terminating peptides; the starting reagent was incubated with N,N-dicyclohexyl carbodiimide (DCC) in the presence of chloroform for a few hours at room temperature. The end-product anhydride is soluble in chloroform, whereas a reaction by-product (dicyclohexyl urea) is not; therefore the anhydride is separated from the undesired by-product by collecting the chloroform and discarding the precipitate DOPE is added to the anhydride in the presence of triethylamine to catalyze the N-acylation reaction; the mixture is incubated overnight at room temperature. The reaction mixture is applied to a preparative thin layer chromatography (TLC) plate to purify N-acetyl-ala-ala-DOPE, the solvent system being chloroform/methanol/water (65/25/4). The lipid band is identified by spraying the plate with water, after which the band is scraped and solubilized in chloroform/methanol (2/1). Lipid is stored under nitrogen at -70C. Specifically, 25 mg (0.12 mmol) of N-acetyl-alanine-alanine was dissolved in 5 ml of dry tetrahydrofuran (THF), and was stirred for 24 hours at room temperature with DCC (25 mg. 0.12 mmol), DOPE (50 mg, 0.26 mmol) and an excess of ethanolamine (ET_3N, 100 ml, 0.7 mmol). The white precipitate of dicyclohexyl urea was filtered through a Celite bed and the filtrate was concentrated. The product was purified was preparative TLC using $CHCl_3$:MeOH:H_2O (60:15:2) to yield 25 mg as a white flaky powder. The product was characterized by TLC and ^3H-NMR (300 MHz, $CDCl_3$). Purity of the preparation was 90% or greater/ The resulting peptide-lipid (N-Ac-Ala-Ala-DOPE) resolved as a single spot by TLC and generated a single peak by reverse-phase HPLC.

B) Chemical Synthesis of 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamido-val-pro-ala-ala-sucMeO (MeO-suc-ala-ala-pro-val-DOPE):

i) p-Nitrophenyl-val-pro-ala-ala-sucMeO ester: To a solution of H-val-pro-ala-ala-sucMeO peptide (540 mg, 1.15 mmol), were added 142 mg (1.38 mmol) of *p*-

nitrophenol, 175 mg (1.38 mmol) of 1,3-dicyclohexylcarbodiimide and a catalytic amount (a few crystals) of 4-dimethylaminopyridine in 10 ml of dry chloroform. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. At this point TLC analysis showed that the reaction had gone to completion. The precipitate, dicyclohexylurea (DCU),from the reaction mixture was filtered using a G-2 funnel and the filtrate concentrated under reduced pressure. The residual material was used in the next step without purification; it was characterized by an R_f 0.43 ($CHCl_3$:MeOH 9:1 v/v).

ii) 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamido-val-pro-ala-ala-sucMeO: To a solution of *p*-nitrophenyl ester of val-pro-ala-ala-sucMeO (600 mg, 1.01 mmol), were added 604 mg (0.81 mmol) of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 82 mg (113 ml, 0.81 mmol) of triethylamine in 20 ml of a mixture of the solvents chloroform:tetrahydrofuran (1:4 v/v). The reaction mixture was stirred under nitrogen atmosphere at room temperature overnight. TLC analysis showed that the reaction had gone to completion. The reaction mixture was concentrated under reduced pressure and passed through activated TMD-8 ion exchange resin in THF:H_2O (9:1 v/v).

The phosphorus positive fractions were pooled and concentrated to get the residual product. The residual material was purified by silica gel column chromatography (column was washed with 5% methanol in chloroform, then eluted with $CHCl_3$:MeOH:NH_4OH 65:25:4 v/v/v), giving 915 mg (95% yield on the basis of DOPE), which on lyophilization gave a white solid: *Rf* 0.76 ($CHCl_3$:MeOH:NH_4OH 65:25:4 v/v/v) and *Rf* 0.43 ($CHCl_3$:MeOH:H_2O 65:25:4 v/v/v).

The lipopeptide molecule gave a positive test for molybdenum reagent and a negative test for ninhydrin reagent. The lipopeptide molecule identity was determined by TLC in two solvent systems: ((i) $CHCl_3$:MeOH:NH_4OH 65:25:4 v/v/v and (ii) $CHCl_3$:MeOH:H_2O 65:25:4 v/v/v). In both solvent systems the lipopeptide gave a single spot and it is >99% pure. The lipopeptide was characterized by NMR and FAB mass analysis. 1H-NMR (CDCl3) some characteristic signals are shown here: d 0.87 (t, 3H, J = 7.15 Hz), 1.27 (40H), 1.56 (4H), 2.0 (8H), 2.23 (t, 4H, J = 7.15 Hz), 5.17 (1H), 5.32 (4H, J = 3.12 Hz). 31P-NMR Spectrum gave single signal. FAB (M+) calculated for

C62H109N5O15P 1195.55 molecular weight, found mass of 1196.8 (MH+) and mass of 1234.9 (MK+).

C) Preparation of peptide-lipid conjugate: DOPE-Ala-Ala-PEG carboxylate

A derivative of DOPE was prepared in which a dialanyl peptide linked the DOPE headgroup to a poly(ethyleneglycol) molecule via amide linkages at each end of the peptide. This is referred to a "DOPE-ala-ala-PEG-carboxylate". For the synthesis, poly(ethyleneglycol) bis(carboxymethyl) (Aldrich) was utilized, a polyethylene glycol with carboxyl groups at each terminus and a molecular weight of 228. To a solution of poly(ethyleneglycol) bis(carboxymethyl) ether (8.5 mg, 0.034 mmole) in 2 ml of chloroform, was added dicyclohexyl carbodiimide (DCC, 5.0 mg, 0.023 mmole). To this solution the peptide-lipid, N-ala-ala- 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (20 mg, 0.023 mmol), was added in chloroform to give a final volume of 3.0 ml. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. At this point thin layer chromatography (TLC) was performed. The precipitate, DCU, from the reaction mixture was filtered using a G-2 funnel and the filtrate concentrated under reduced pressure. TLC indicated the necessity to further purify the preparation. The remaining material was further purified by silica gel column chromatography, as in section B of this Example, to remove residual starting material and yielded 6.5 mg of purified material (>99%) with an Rf of approximately 0.28 on a silica gel TLC plate run in the solvent $CHCl_3$/methanol/NH_4OH (65/25/4). As expected this purified product was more polar than the starting peptide-lipid. By TLC this product gave a single spot and >99% purity. The product was also characterized FAB mass analysis. The calculated FAB (M+) was 996 and a mass of 1134.6 (MK+) was observed.

Example 2

Elastase and proteinase K-mediated cleavage of N-Ac-ala-ala-DOPE

Cleavage of N-Ac-ala-ala-DOPE to DOPE by elastase was monitored by thin layer chromatography (TLC). The chemical structure of N-Ac-ala-ala-DOPE and its postulated scheme of conversion to DOPE by enzymatic cleavage is shown in Fig. 1. 100-200 nmol of N-Ac-AA-DOPE SUVs were incubated with 1 mg enzyme in 0.1 ml overnight at 37oC. Human leukocyte elastase was purchased from Calbiochem (San

Diego, CA). Lipid was extracted by organic phase separation (Bligh and Dyer, 1959) twice. Collected lipid was dried under N2 stream and exposed to vacuum for 4 hours-overnight. Samples were resuspended in chloroform and spotted onto TLC plates. TLC was run using chloroform/methanol/water (65:25:4), air dried, sprayed with molybdenate blue, and charred on a hot plate. Treatment of N-Ac-AA-DOPE liposomes with elastase generated a product corresponding to DOPE, whereas untreated N-Ac-AA-DOPE showed no change (Fig. 2A). Therefore elastase recognized N-Ac-AA-DOPE and cleaved the dipeptide to yield DOPE.

Several proteases were tested to determine whether an enzyme with similar substrate specificity could be used as a model for elastase mediated cleavage of *N*-Ac-AA-DOPE. Proteinase K is a serine protease that, similarly to elastase, can cleave at peptide bonds C-terminal to aliphatic residues. Upon incubation of *N*-Ac-AA-DOPE liposomes with proteinase K the peptide-lipid was cleaved and DOPE was generated (Fig. 2B).

Subsequently, proteinase K, which has a similar substrate specificity to elastase, was used to model the elastase effect because of the lower cost of the enzyme. Referring to Fig. 3, samples A and C contained pure N-acetyl-ala-ala-DOPE and pure DOPE, respectively; sample B consisted of multilamellar liposomes, prepared as described hereinbelow, composed of pure N-acetyl-ala-ala-DOPE (2 µmole total phospholipid), which were incubated in 200 µl of 154 mM NaCl, 10 mM TES, 0.1 mM EDTA at pH 7.4 and 37 °C for two hours in the presence of 1 mg of proteinase K. After the first incubation, another equal amount of proteinase K was added and the sample was incubated for another 2 hours. All three samples were dissolved into a final concentration of 5% deoxycholate, 50 mM EDTA and 10 mM HEPES at pH 8 for proton decoupled ^{31}P NMR. Integration showed that approximately 20% of the N-acetyl-ala-ala-DOPE was converted to pure DOPE in sample B.

The conversion of *N*-Ac-AA-DOPE to DOPE was also monitored by ^{31}P-NMR analysis. *N*-Ac-AA-DOPE LUVs were prepared and treated with or without proteinase K (1.5 mg protein/100 nmol lipid) overnight at 37°C. Samples were mixed with buffer (10% deoxycholate, 100mM EDTA, 20mM Hepes) and deuterium oxide (Cambridge Isotope Laboratories, Woburn, MA) (1:4:2) and transferred to 5mm NMR tubes. Samples were

monitored at room temperature in a Bruker AC300 spectrometer operating at 121.5 MHz, with 110ms 90° radio frequency pulse for proton decoupling and set to 2 sec interpulse delay to avoid signal saturation. Sweep width was set at 50kHz. 1 Hz line broadening was applied to all spectra. N-Ac-AA-DOPE liposomes treated with proteinase K (1.5 mg protease/100 nmol lipid) resulted in the appearance of a peak 0.3 ppm upfield from *N*-Ac-AA-DOPE, corresponding with pure DOPE.

Elastase and proteinase K mediated cleavage of *N*-Ac-AA-DOPE was quantitated using liposomes composed of *N*-Ac-AA-DOPE and DOTAP, a positively charged lipid. DOTAP was included to provide a counterbalancing positive charge, and was used as a standard by which different samples could be normalized and compared. After treatment with elastase or proteinase K the reduction in the amount of *N*-Ac-AA-DOPE was monitored by HPLC (Fig. 4). Liposomes composed of DOTAP/*N*-Ac-AA-DOPE (1:1) were incubated with enzyme under given conditions. Lipid was extracted by the Bligh-Dyer procedure twice.

Collected lipid was dried under a N_2 stream and exposed to vacuum for 4 hours-overnight. Samples were resuspended in 100% ethanol and injected in 30 ul aliquots into Spherisorb silica columns (150 x 4.6mm, 0.3 um, Keystone Scientific). HPLC was performed using a hexane:isopropanol:water:TFA mobile phase. Hexane and TFA were held constant at 37% and 0.2%, respectively. The *N*-Ac-AA-DOPE peak was detected using a gradient of 59-55% isopropanol:4-8% water. Flow rate was 1.5 ml/min, column temperature was set at 45°C, and peaks were detected by a UV detector set at 205nm. Lipid peaks were quantitated in comparison to standard curves generated by injecting 5-200 nmol of DOTAP or *N*-Ac-AA-DOPE and monitoring 205nm signal. Percent cleavage was calculated by normalizing peaks to DOTAP, then determining the decrease in *N*-Ac-AA-DOPE peak size relative to starting amounts.

Both elastase and proteinase K cleaved *N*-Ac-AA-DOPE to a similar extent (Fig. 4). To verify that the cleavage of *N*-Ac-AA-DOPE was due to proteinase K enzymatic activity, liposomes were treated with heat inactivated proteinase K. Proteinase K was inactivated by heating at 95°C for 1 hour, after which the enzyme was incapable of

cleaving the chromogenic substrate *N*-Ac-AAA-pNA. Treatment of DOTAP/*N*-Ac-AA-DOPE liposomes with heat inactivated proteinase K did not result in any cleavage of *N*-Ac-AA-DOPE (Fig. 4), indicating the requirement for active proteinase K. Since proteinase K has been shown to share substrate specificity with elastase and is considerably less costly than human leukocyte elastase, several experiments were conducted with proteinase K. Later experiments on the effects of human leukocyte elastase on N-Ac-ala-ala-DOPE used thin layer chromatography ("TLC"), a high sensitivity method, to detect the product of enzyme activity. In these experiments liposomes, prepared as described hereinbelow, were composed of 100% N-Acetyl-ala-ala-DOPE and were then extruded to approximately 0.2 microns diameter/ Fifty micrograms of liposomal lipid was incubated alone or with one unit of HLE overnight at 37ºC.

Example 3

Cleavage of MeO-suc-ala-ala-pro-val-DOPE by human leukocyte elastase (HLE)

A) HLE dose titration

To determine if the peptide-lipid MeO-suc-ala-ala-pro-val-DOPE is also a suitable substrate for elastase mediated cleavage 50 nmol of MeO-suc-ala-ala-pro-val-DOPE liposomes (SUVs) were incubated with 0, 2.5, 5, 10, or 20ug HLE (from Calbiochem; 20 units/mg protein; 1 unit = amount of enzyme that will hydrolyze 1.0 umol of MeO-suc-ala-ala-pro-val-pNA per min at 25 degrees C, pH 8.0) overnight at 37 degrees C in 50 ul volume of 10mM TES/ 154 mM NaCl/ 0.1mM EDTA, pH 7.4, containing 1.5mM Ca and 1.5 mM Mg.

Lipid was extracted using the Bligh-Dyer technique (chloroform/methanol/water : 2/1.7/1), dried under nitrogen, placed under high vacuum for ~ 3 hours. Samples were resuspended in 5 ul chloroform and spotted onto TLC plates. 20ug of pure DOPE was also spotted for comparison purposes. TLC solvent system was chloroform/methanol/ammonium hydroxide (65/25/5). Plates were air dried, sprayed with molybdenate blue, then charred at 180 degrees C.

As shown in Fig. 5A, between 5 -40 micrograms of HLE were effective in cleaving the AAPV-PE (compare lanes 2,3,5 & 6 with lane 4 & 8, and with the proteinase K control, lane 7).

B) Kinetics of HLE-mediated cleavage of MeO-suc-ala-ala-pro-val-DOPE: MeO-suc-ala-ala-pro-val-DOPE liposomes were incubated with 0 or 5 ug HLE for 1, 2, 4 hours, or overnight and processed as above. The peptide was cleaved by HLE in as little as 1 hour at 37 degrees C (Fig. 5B, lane 2), suggesting that the cleavage of MeO-suc-ala-ala-pro-val-DOPE occurs within a physiologically relevant time frame.

C) Cleavage of MeO-suc-ala-ala-pro-val-DOPE by human neutrophil granule proteins

Since elastase is produced by activated neutrophils the cleavage of MeO-suc-ala-ala-pro-val-DOPE by unpurified granule proteins was monitored to mimic more closely the in vivo situation. Neutrophils were obtained from human whole blood by standard procedures employing density centrifugation. Granules were isolated from these neutrophils by centrifugation following nitrogen cavitation of cells, again following established procedures. Protein concentration of neutrophil granules was determined after repeated freeze-thawing of granules to release proteases.

Fifty nmol MeO-suc-ala-ala-pro-val-DOPE liposomes (SUVs) were incubated with 0, 2.5, 5, 10, or 20ug neutrophil granule proteins overnight at 37 degrees C in 50 ul volume of 10mM TES/ 154 mM NaCl/ 0.1mM EDTA, pH 7.4, containing 1.5mM Ca and 1.5 mM Mg. Samples were processed as described above. The results shown in Fig. 5C indicate that 2.5 μg of neutrophil granule proteins were sufficient to detect cleavage of MeO-suc-ala-ala-pro-val-DOPE to DOPE, suggesting that crude neutrophil granule proteins can convert the peptide-lipid to DOPE, and therefore liposomes containing this peptide-lipid can be activated to fuse under physiological conditions.

The conversion of MeO-suc-AAPV-DOPE to DOPE was also quantitated by ^{31}P-NMR. Since subsequent experiments employed liposomes containing both DODAP and MeO-suc-AAPV-DOPE, vesicles prepared at a 1:1 (mol:mol) ratio of these two

components were prepared by freeze-thaw/extrusion method as described above, and incubated at 37ºC for 2 hours with increasing amounts of elastase (0 micrograms to 5 micrograms elastase/100 nmol lipid). ^{31}P-NMR analysis demonstrated an elastase concentration dependent cleavage of MeO-suc-AAPV-DOPE and appearance of DOPE (Fig. 6, solid line). A small shoulder that may indicate an incomplete peptide cleavage product was also observed near the original peptide-lipid peak. Treatment with 5 micrograms of elastase/100 nmol lipid yielded 20% DOPE. Longer incubation may have lead to further digestion, though multiple lamellae and/or the surface charge of the liposome may limit the ultimate amount digested. The maximum exposed peptide-lipid for intact liposomes would occur with unilamellar vesicles. Assuming only the outer leaflet peptide-lipid in unilamellar vesicles is available for digestion, the minimal percentage conversion of exposed MeO-suc-AAPV-DOPE to DOPE in the outer leaflet lipid of this preparation was 40% (Fig. 6, dotted line). If the average number of lamellae were greater than one, the percentage conversion was even higher. The ratio of encapsulated volume to total lipid would appear to indicate an average of approximately 2.5 lamellae per vesicle for this preparation which would indicate that 100% of available peptide-lipid had been cleaved under these conditions. The concentration of elastase to produce this amount of cleavage (12.5 micrograms elastase/ml) is less than the effective concentration of elastase activity found in the epithelial lining fluid from patients with cystic fibrosis (Birrer et al, 1994). The contents from human neutrophil granules also cleaved MeO-suc-AAPV-DOPE and generated DOPE. The number of neutrophils required to observe this effect was less than that observed in epithelial lining fluid from cystic fibrosis patients (Birrer et al., 1994), indicating the amount of elastase required to cleave MeO-suc-AAPV-DOPE is within concentrations that are physiologically or therapeutically relevant

Example 4

Enzyme-mediated cleavage of N-Acetyl-ala-ala-PE.: To compare the effect of different proteolytic enzymes on the cleavage of N-acetyl-ala-ala-DOPE, small unilamellar liposomes composed of DOTAP and N-acetyl-ala-ala-PE (1:1 molar ratio, 100 nmole), prepared as described hereinabove, were incubated overnight at 37 degrees C. with either 0.5 mg (experiment 1) or 1.0 mg (experiment 2) of: S. caespitosus protease S.

griseus ("pronase"), peptidase, chymotrypsin, trypsin, protease type I, proteinase K, or with no enzyme added.

Subsequently, lipid was extracted from these preparations by organic phase separation, and dried under vacuum. The dried lipid was suspended in ethanol, and the resulting lipidic solution was then injected into a normal phase silica column. Analysis was by HPLC, as described above. Lipid peaks were quantitated, and the amount of lipid cleavage was calculated as a percentage of the starting lipid. Fig. 7 shows that both proteinase K, with a known cleavage site, and the nonspecific mixture of proteases found in pronase, effected cleavage of N-Ac-ala-ala-DOPE from the liposomes, and the greater release being obtained with the increased enzyme concentrations. Other proteases, however, did not cleave the N-Ac-ala-ala-DOPE from the liposomes.

Example 5

Liposome Preparation

NBD/Rh labeled or unlabeled large unilamellar vesicles (LUVs) were prepared as described before (Mayer et al.). Briefly, the lipid mixture in chloroform was dried under a nitrogen stream to a thin film, which was then left under vacuum overnight to remove residual solvent. The lipid film was hydrated with TES buffered saline (10 mM TES, 0.1 mM EDTA, 154 mM NaCl, pH 7.4). Brief vortexing was applied to ensure complete hydration. After ten cycles of freeze/thaw in liquid nitrogen/room temperature water bath, the sample was extruded ten times through 0.1 μm polycarbonate membrane filter (Poretics Corp., Livermore, CA). The liposomes were stored at 4°C. Multilamellar vesicles were prepared by hydrating the dried lipid film.

The phospholipid concentration of each liposome preparation was determined by phosphate assay (Bartlett). The approximately 0.1 μm size of the liposomes was confirmed on a Nicomp submicron particle sizer (Nicomp Instruments, Inc., Goleta, CA) using quasi-elastic light scattering.

Example 6

Preparation of Resealed and Unsealed Human Erythrocyte Ghosts

Resealed ghosts are referred to as erythrocyte ghosts unless otherwise specified, and were prepared as previously described (see Williamson et al.; Clague et al., the contents of which are incorporated herein by reference). Briefly, fresh human blood was washed several times with cold 10 mM TES buffered saline to remove plasma and white cells. Then 2 ml of washed erythrocytes (50% hematocrit) were pre-swelled in cold hypotonic solution containing 8 ml H_2O and 9.6 ml 10 mM TES buffered saline, and then pelleted at 850xg for 5 minutes. The pellet was resuspended in 40 ml cold lysis buffer (10 mM Tris, 0.1% bovine serum albumen (BSA), 2 mM $MgCl_2$, and 0.1 mM EGTA) and incubated on ice for at least 2 minutes. After addition of 4.5 ml 10x resealing buffer (1.22 M NaCl, 30 mM KCl, 0.15 M Na_2HPO_4, 50 mM KH_2PO_4, and 2 mM $MgCl_2$), the sample was incubated at 37°C for 40 minutes. The resealed ghosts were pelleted at 1750xg for 10 minutes and washed several times until no hemoglobin could be observed in the supernatant. The ghosts were stored at 4°C and used within one week. A prothrombinase assay was used to test for maintenance of phospholipid asymmetry in the ghosts resulting from this method of preparation, as described below.

Example 7

Prothrombinase Assay

The phospholipid asymmetry of the erythrocyte membranes was measured by a prothrombinase assay, which detects the presence of PS (phosphoserine) in the outer monolayer of the membrane. The assay was performed as previously described (Wilson et al., 1993, the contents of which are incorporated herein by reference) with some modifications. Briefly, 4×10^{15} erythrocyte ghosts were incubated at 37°C for 3 minutes in Tris buffer (50 mM Tris-HCl, 120 mM NaCl, pH 7.4) containing 6 mM $CaCl_2$, 0.33 unit ml^{-1} factor V/Va, 0.33 unit ml^{-1} factor Xa, and 1.3 unit ml^{-1} prothrombinase; the total reaction volume was 1 ml. After 15 mM EDTA was added to stop the reaction, the cells were pelleted by centrifugation. About 900 µl supernatant was mixed with 100 µl chromogenic substrate, sarcosine-Pro-Arg-*p*-nitroanilide (500 µM) and OD_{405} was measured kinetically. The rates (ΔOD/min) of intact erythrocytes and unsealed erythrocyte ghosts were taken as 0% and 100% accessible PS, respectively. Using this scale, the accessible PS on the resealed ghosts was found to be about 16%.

Example 8

Design of fusion-triggerable liposomes containing *N*-Ac-AA-DOPE

The threshold of fusogenicity was determined by preparing liposomes with increasing amounts of PE transesterified from egg PC. This PE was preferred over DOPE because of its higher H_{II} transition temperature (~37°C vs. 10°C, respectively), which aids in the preparation of stable liposomes yet does not inhibit fusion. DOTAP was chosen as the positively charged lipid. Fusion assays were performed for DOTAP/*N*-Ac-AA-DOPE/PE liposomes containing the fluorescent membrane probes *N*-NBD-PE ((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) PE)and *N*-Rho-PE (N-lissamineorhoadmine B sulfonyl (PE))and inversely varying amounts of *N*-Ac-AA-DOPE and PE. These liposomes were monitored for lipid mixing with either unlabeled target liposomes or for lipid mixing and binding with RBC ghosts. Lipid mixing between NBD/Rh labeled liposomes and unlabeled ghosts was measured in 10 mM TES buffered saline by the NBD/Rh resonance energy transfer (RET) assay (Struck et al., the contents of which are incorporated herein by reference).

Liposomes were prepared with 1 mol% N-NBD-PE and 1 mol% N-Rho-PE, which results in quenching of the N-NBD-PE fluorescence signal. Membrane fusion results in probe diffusion and relief from self-quenching, which is monitored as an increase in N-NBD-PE fluorescence. Liposome-liposome lipid mixing was initiated by addition of 10 nmol of fluorescently labeled liposomes to 90 nmol unlabeled liposomes in microcentrifuge tubes containing 1 ml of TES/NaCl/EDTA buffer with 1.5mM Ca++/1.5mM Mg++. For fusion with cells 1x108 RBC ghosts were substituted for unlabeled liposomes. All samples were shaken in an Eppendorf Thermomixer (Brinkmann Instruments, Inc., Westbury, NY), 700 rpm/min, during the 37oC incubation for 30min. N-NBD-PE fluorescence was monitored in a T-format PTI Alphascan spectrafluorometer (Princeton, NJ) with a xenon short arc lamp using 450 nm excitation/530nm emission wavelengths and 5nm slitwidths. 450 nm band pass and 500nm cutoff filters were utilized for excitation and emission light paths, respectively, to reduce stray light. Maximal fluorescence dequenching was determined by addition of 0.1% C12E8 detergent (octaethylene glycol monododecyl ether).

The threshold for fusogenicity depends upon the target in question. Liposomes composed of DOTAP (20%) and PE (80%) fused with both target liposomes and RBC ghosts. Inclusion of 10 mol% N-Ac-AA-DOPE with a corresponding decrease in PE to 70 mol% generated liposomes that were still capable of fusing with PE/PS liposomes but not PC/PS liposomes (Fig. 8A). The requirements for membrane fusion with RBC ghosts appeared to be more stringent, with inclusion of 5 mol% N-Ac-AA-DOPE inhibiting both the lipid mixing and the binding significantly (Fig. 8B). Defining the different threshold of fusion for different targets creates a gradient of sensitivity for fusion that can be used to determine optimum conditions for activating N-Ac-AA-DOPE containing liposomes to fuse. As PE/PS liposomes appeared to be the most sensitive target, we focused on a composition of DOTAP/N-Ac-AA-DOPE/PE liposomes that could be activated to fuse. The threshold of PE content appeared to be between 65-70 mol%. In order to create a liposome that is not initially highly positively charged, DOTAP and N-Ac-AA-DOPE were added in equivalent amounts to yield liposomes composed of DOTAP/N-Ac-AA-DOPE/PE in a 15/15/70 mol ratio.

These results suggests that the threshold PE content is between 65-70 mol% for fusion with PS/PE liposomes, but 70-80% for fusion with erythrocyte ghosts.

Example 9

Concentration-dependence and time-dependence of proteinase K activity

Having established the liposome compositions close to the threshold for fusion, the concentration and time-dependence of proteinase K activation of fusion was determined. Large unilamellar liposomes were prepared and fluorescently labeled, as described hereinabove, so as to contain DOTAP, N-acetyl-ala-ala-PE and tPE ("transphosphatidylated PE," PE prepared from egg PC by transphosphatidylation reaction; i.e., phospholipase D, treatment of egg PC in presence of phosphoethanolamine) in a 15:15:70 mole % ratio. For fusion experiments, 1 mole% of N-NBD-PE and 1 mole % of N-Rho-PE were added as fluorescence probes. For cleavage experiments, liposomes without the fluorescent probes were used.

Aliquots of these liposomes were then incubated at 37 degrees C. overnight with: 0, 0.1, 0.25, 0.5 or 1.0 mg of proteinase K (100 nmoles lipid/1mg protein). Subsequently, aliquots of these effector liposome preparations were incubated with unlabeled PE/PS acceptor liposomes, at a ratio of 1:10 effector liposomes:acceptor liposomes. The degree of lipid mixing between effector and acceptor liposomes, assessed as described hereinabove, was then determined as a measure of interliposome fusion

Large unilamellar liposomes were prepared as described hereinabove to contain DOTAP and N-acetyl-ala-ala-PE in a 1:1 molar ratio. Lipid extraction and HPLC analysis was then performed, as described hereinabove, to assess the extent of lipid cleavage. The reliance of fusion activation upon enzyme cleavage of *N*-Ac-AA-DOPE was further assessed by examining the concentration and time dependencies of both events. DOTAP/*N*-Ac-AA-DOPE/PE liposomes were either incubated with 0, 0.1, 0.25, 0.5, and 1 mg proteinase K/100nmol lipid overnight, or with 1 mg proteinase K/100nmol lipid for 1, 2, 4 hours or overnight. These liposomes were monitored for *N*-Ac-AA-DOPE cleavage by HPLC or for lipid mixing with acceptor liposomes by *N*-NBD-PE

fluorescence dequenching. A similar concentration dependence was evident for both *N*-Ac-AA-DOPE cleavage and liposome fusion (Fig. 0A). Treatment with 0.5 or 1 mg proteinase K yielded apparently maximal cleavage and fusion activity. Only background levels of both activities were observed when 0 or 0.1 mg of the enzyme were used. The kinetics of proteinase K mediated cleavage and fusion activation were also correlated, with overnight incubation giving the highest amount of cleavage and lipid mixing (Fig. 9B, and date described in Example 2). These results further support the contention that the activation of fusion of DOTAP/*N*-Ac-AA-DOPE/PE liposomes is due to enzymatic cleavage of *N*-Ac-AA-DOPE.

As shown in Fig. 8A, between 0.5 - 1.0 mg of proteinase K effected maximal cleavage and fusion activity, and that lipid mixing is a good indicator for monitoring NAc-ala-ala-DOPE cleavage. Fig. 8B shows no significant differences when liposomes are incubated with proteinase K for a period of between 1 and 4 hours, and that maximal release and fusion activity, as measured by both methods, is maximal after an overnight incubation.

Example 10

Activation of liposome-liposome lipid mixing by elastase and proteinase K cleavage

Since elastase and proteinase K were capable of cleaving *N*-Ac-AA-DOPE to DOPE (Figs. 2 & 4) both enzymes were tested for their ability to activate DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes to fuse. These liposomes were treated overnight at 37°C with elastase, or proteinase K, or without either enzyme, after which liposomes were incubated with PE/PS liposomes and lipid mixing monitored by relief of *N*-NBD-PE fluorescence quenching. Liposomes were incubated with protease at a 1 mg protease/100 nmol lipid/0.1 ml buffer ratio, unless otherwise stated. This concentration of proteinase K was found to have comparable activity, within an order of magnitude, with that of elastase in rheumatoid arthritis synovial fluid (Al-Haik et al., 1984, Agents Actions 15:436-442; and our unpublished data]. Mixtures were incubated at 37°C in microcentrifuge tubes with constant shaking in an Eppendorf Thermomixer, 700

rpm/min. Treated liposomes were then assayed for *N*-Ac-AA-DOPE cleavage by HPLC, as described above. For fusion experiments liposomes containing fluorescent membrane probes were treated with protease and then the concentrations of liposomes were determined by monitoring direct *N*-Rho-PE fluorescence (550ex/590em) and comparing with a known amount of stock liposomes. Aliquots of these fluorescently labeled protease treated liposomes were incubated with unlabeled target liposomes or cells and lipid mixing was determined as described above.

Treatment by either enzyme resulted in a greater extent of lipid mixing over that of untreated liposomes (Fig. 10). This result, coupled with the shared substrate specificity of proteinase K and elastase, suggests proteinase K activation serves as a suitable substitute for elastase to characterize the fusion activation of *N*-Ac-AA-DOPE containing liposomes.

A causal relationship between cleavage of the *N*-Ac-AA-DOPE peptide-lipid and fusion activation of DOTAP/*N*-Ac-AA-DOPE/PE liposomes was studied using heat inactivated proteinase K. DOTAP/*N*-Ac-AA-DOPE/PE (15/15/70 mol%) liposomes containing the fluorescent membrane probes *N*-NBD-PE and *N*-Rho-PE were incubated overnight at 37°C with active or heat inactivated proteinase K, after which an aliquot of the liposomes was incubated with unlabeled PS/PE acceptor liposomes to monitor the extent of lipid mixing. Treatment of DOTAP/*N*-Ac-AA-DOPE/PE liposomes with active proteinase K resulted in ~30% fluorescence dequenching, a six-fold increase in lipid mixing over the untreated liposomes (Fig. 11). However, treatment with an identical amount of the heat inactivated enzyme did not activate liposomes to fuse (Fig. 11). Therefore, enzymatic activity is essential for the liposomes to become fusogenic, indicating *N*-Ac-AA-DOPE cleavage is crucial for triggering the fusogenic potential.

Example 11

Activation of DOTAP/*N*-Ac-AA-DOPE/PE fusion with RBC ghosts

Since DOTAP/*N*-Ac-AA-DOPE/PE liposomes could be activated to fuse with target liposomes after enzymatic cleavage, we determined if activated fusion of *N*-Ac-AA-DOPE containing liposomes with cell membranes could also be observed. As fusion

with RBC ghosts (Fig. 8B) appeared to exhibit a different threshold of fusogenicity than liposomes (Fig. 8A), we prepared DOTAP/*N*-Ac-AA-DOPE/PE liposomes at a 20/10/70 mol ratio. The overall positive charge of these liposomes improves the binding to cell membranes, relative to the 15/15/70 composition, without permitting the liposomes to fuse with cell membranes in the absence of an activating trigger (Fig. 8B). After an overnight, 37°C, incubation of these liposomes with proteinase K, lipid mixing with RBC ghosts was observed in the presence of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) (Fig. 12, with Proteinase K). The activity of residual proteinase K transferred from the initial incubation was negligible (Fig. 12, Proteinase K control). Specific activation of DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) fusion with RBC ghosts was also observed under continuous kinetics conditions. Only liposomes pretreated with proteinase K were capable of fusing with RBC ghosts while untreated liposomes did not (Fig. 13). The addition of active proteinase K to untreated liposomes also did not induce fluorescence dequenching (Fig. 13, curve c), indicating the observed increase for proteinase K treated DOTAP/*N*-Ac-AA-DOPE/PE liposomes was due to specific fusion activation.

To determine if the lipid mixing observed after proteinase K activation was due to true fusion of liposomes with cell membranes and not potential artifacts of the lipid mixing assay, such as membrane probe exchange or hemifusion between outer leaflet membranes, DOTAP/*N*-Ac-AA-DOPE/PE (20/10/70 mol%) liposomes were loaded with 10,000 MW fluorescent aqueous probe TX-red dextran. Liposomes were then treated with proteinase K and incubated with RBC ghosts. After washing extensively to remove unbound liposomes the RBC ghosts were observed under fluorescence microscopy. The pellet was resuspended in 0.1 ml buffer and observed under an Olympus BH-2 fluorescence microscope (Olympus Corp., Lake Success, NY) using an apochromat 40x oil (N.A. 1.00) objective. TX-red fluorescence was excited by a xenon lamp transmitted through a green excitation cube (580nm dichroic mirror, 545nm excitation filter). Non-fluorescent images were observed with transmitted light Nomarski differential interference contrast microscopy.

Bright diffuse fluorescence could be observed in a portion of the ghosts (Fig. 14), indicating complete fusion occurred between liposomes and certain ghosts with subsequent transfer of the fluorescent aqueous probe. Differences in fluorescence levels may be due to differences in the number of liposomes fusing with a single ghost. The observed fluorescence does not appear to be due to non-specific uptake of dextran out of leaky liposomes, as incubation of RBC ghosts with unlabeled liposomes and free TX-red dextran did not result in observable aqueous probe labeling (Fig. 14). Thus DOTAP/*N*-Ac-AA-DOPE/PE liposomes can be activated by enzymatic cleavage of the peptide-lipid to fuse with cell membranes and deliver their aqueous contents.

Example 12

Cells

HL60 human leukemia and ECV304 human cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). HL60 cells were passaged as suspension cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Adherent ECV304 cells were grown in Medium 199 supplemented with 10% heat-inactivated FBS. Greater than 98% viability was observed during routine tissue culture. RPMI 1640, FBS and Hanks' Balanced Salt Solution (HBSS) were purchased from Life Technologies, (Gaithersburg. MD).

Example 13

Activation of MeO-suc-ala-ala-pro-val-DOPE containing liposomes by HLE for enhanced binding/fusion to HL60 cells

100 nmol of DOTAP/ AAPV-PE (3:1; 1:1; or 1:3) were incubated with or without HLE (5ug/100nmol lipid) in 100 ul for 2 hours at 37C, pH 7.4 with constant shaking. Afterwards 10nmol of liposomes with or without HLE pretreatment were incubated with 1-2 x 10^6 HL60 (human leukemia) cells at pH 7.4 or 4. Samples were incubated 2 hours at 37C with constant shaking. After incubation, cells were washed once, resuspended in 0.5 ml, and transferred to wells of Falcon 24 well plate (PRIMARIA {Trademark], Becton-Dickinson Co., Lincoln Park, NJ). Fluorescence was monitored with or without detergent in a Cytofluor plate reader (Perseptive Biosystems, Framingham, MA).

Liposome input (no cells) were not washed and were transferred directly to the wells of 24 well plates.

The results of a series of experiments are described below in Table 1 In a first series of experiments, liposomes exhibited higher binding at pH 4.0. Binding also appeared to be higher after HLE pretreatment. In addition, DODAP/AAPV-PE (1:1) may possibly exhibit higher fluorescence dequenching after HLE treatment, suggesting lipid mixing between liposomes and cells had occurred.

	% lipo bound		% NBD FDQ	
DODAP/AAPV-PE	**pH 7.4**	**pH 4.0**	**pH 7.4**	**pH 4.0**
1:3 - HLE	2.68	53.58	104.22	33.13
1:3 + HLE	0.74	70.34	-32.43	32.53
1:1 - HLE	2.37	56.95	59.94	23.86
1:1 + HLE	2.87	85.91	77.42	44.81
3:1 - HLE	6.76	67.88	-5.05	64.52
3:1 + HLE	7.78	80.82	43.43	69.04

Three separate experiments also displayed enhanced binding of DODAP/AAPV-PE (1:1) to HL60 cells. One showed an enhancement from 64% to 86% fluorescence dequenching. In terms of total number of liposomes fused, HLE pretreatment appeared to enhance lipid mixing in all three experiments.

	w/o HLE	w/ HLE
% bound	20.29	45.22
# bound	1.22E+10	2.72E+10
% lipid mixing	48.06	44.24
# lipid mixed	5.87E+11	1.20E+12

	w/o HLE	w/ HLE
% bound	7.72	33.17
# lipos bound	4.60E+09	2.00E+10
% lipid mixing	57.12	25.89
# lipid mixing	2.65E+11	5.17E+11

	w/o HLE	w/ HLE
% bound	38.09	46.89
# Lipos bound	2.29E+10	3.08E+10
% lipid mixing	63.74	86.25
# lipid mixed	1.46E+12	2.43E+12

Example 14.

Binding and lipid mixing of liposomes to HL60 cells

In another series of experiments, lipid mixing was monitored by the *N*-NBD-PE/*N*-Rho-PE resonance energy transfer assay, as described [Struck et al., 1981]. Liposomes were prepared with 0.75 mol% *N*-NBD-PE and 0.75 mol% *N*-Rho-PE, which results in quenching of the *N*-NBD-PE fluorescence signal. Membrane fusion results in probe diffusion and relief from self-quenching, which is monitored as an increase in *N*-NBD-PE fluorescence. DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) liposomes were incubated in TES/NaCl/EDTA buffer with or without elastase (5 • g/100 nmol lipid, 250 • M lipid concentration) for 2 hours at 37°C, pH 7.4. HL60 cells were washed with TES/NaCl/EDTA buffer and incubated with liposomes ($1x10^6$ cells, 10 nmol liposome) in 200 • l TES/NaCl/EDTA buffer. Samples were either at pH 7.4 or adjusted to pH 5 by

the addition of dilute HCl. All samples were shaken in an Eppendorf Thermomixer (Brinkmann Instruments, Inc., Westbury, NY), 700 rpm/min, for 30 min at 37°C. There was no reduction in cell viability following this procedure, as detected by trypan blue exclusion (unpublished data). Cells were then washed with TES/NaCl/EDTA buffer, pH 7.4, and transferred to Falcon 24 well plates (Becton Dickinson, Lincoln Park, NJ). Fluorescence was monitored in a Cytofluor II multiwell fluorescence plate reader (Perseptive Biosystems, Framingham, MA) with a quartz halogen lamp using 450 nm excitation/530nm emission or 560 nm excitation/620 nm emission wavelengths for *N*-NBD-PE or *N*-Rho-PE fluorescence, respectively. Liposome binding was determined as the amount of *N*-Rho-PE fluorescence associated with washed cells relative to total fluorescence of liposomes added. This percentage was converted to number of liposomes bound by multiplying by the number of liposomes added (assuming all liposomes were 100 nm in diameter and 10^5 lipid molecules/0.1 um diameter liposome. Therefore 6.02×10^{10} liposomes of 0.1 um diameter were added per sample). The process of lipid mixing is measured by the fluorescence dequenching (FDQ) of the NBD fluorophore. The % fluorescence dequenching (FDQ) was calculated by the following formula:

$$[[(F_t/F_{max\ cells})-(F_{o\ alone}/F_{max\ alone})]/[1-(F_{o\ alone}/F_{max\ alone})]] \times 100$$

where F_t = *N*-NBD-PE fluorescence of liposomes incubated with cells at a given time, $F_{o\ alone}$ = initial *N*-NBD-PE fluorescence of liposomes only, $F_{max\ cells}$ and $F_{max\ alone}$ = maximal *N*-NBD-PE fluorescence of liposomes incubated either with cells or alone, respectively, as determined by addition of 0.5% C12E8

FDQ was assumed to result from all-or-none lipid mixing of liposomes with cells. Therefore % FDQ could be converted to number of liposomes mixed by simple multiplication of the total. This was done to take into account both the enhancement of binding and the lipid mixing after elastase activation.

Example 15

Optimum DODAP/MeO-suc-AAPV-DOPE composition for binding and lipid mixing with HL60 cells

MeO-suc-AAPV-DOPE containing liposomes were designed to deliver their contents after binding, endocytic internalization, and fusion with and/or disruption of the endosomal membrane. DODAP was chosen instead of DOTAP (Pak et al., 1998) because only 20% of the DODAP population is positively charged at pH 7.4 [Bailey and Cullis, 1994). This allows a more complete peptide hydrolysis by elastase so that we can model the more general case of cleavage on the liposome surface that is not affected by the high charge of the enzyme. For other enzymatic activators, it may be possible to utilize a permanently positively charged lipid that would allow the liposome to become positively charged at physiological pH after peptide cleavage such that extracellular binding would be triggered. By contrast, the tertiary amine of DODAP would be fully protonated at pH 5, suggesting liposomes containing DODAP would undergo stronger interaction with negatively charged cell membranes within the low pH environment of the endocytic compartment.

To determine the optimum combination of DODAP and MeO-suc-AAPV-DOPE for triggerable binding and lipid mixing with cells the two lipids were formulated into liposomes at different ratios. DODAP/MeO-suc-AAPV-DOPE liposomes prepared at 1:3, 1:1, and 3:1 mol ratios were pretreated with or without elastase and incubated with HL60 cells under low pH conditions to promote DODAP mediated binding to cells. Only DODAP/MeO-suc-AAPV$_2$-DOPE liposomes at a 1:1 mol ratio exhibited an elastase dependent increase in binding and lipid mixing with HL60 cells (Fig. 15), possibly as a result of increased positive charge after enzymatic cleavage. The amount of DODAP in 1:3 liposomes was insufficient to mediate binding to cells, even at pH 5 after elastase treatment. By contrast, DODAP/MeO-suc-AAPV-DOPE (3:1 mol ratio) liposomes were able to bind to cells with or without elastase treatment, reflecting the greater amount of DODAP in these liposomes. These liposomes were also able to lipid mix with cells without elastase activation. The DODAP/MeO-suc-AAPV-DOPE 1:1 mol ratio liposomal formulation was chosen for all further studies in order to develop a delivery system that can be triggered by enzymatic cleavage.

Example 16

Elastase-activated binding and lipid mixing of DODAP/MeO-suc-AAPV-DOPE liposomes with HL60 cells.

DODAP was included in liposomes with MeO-suc-AAPV-DOPE to enhance binding with cells under low pH conditions. To determine whether the pH dependence of DODAP mediated binding is within physiological levels, DODAP/MeO-suc-AAPV-DOPE (1:1) liposomes were pretreated with elastase and incubated with HL60 cells at different pH. Enhanced binding and lipid mixing of elastase pretreated liposomes with HL60 cells were observed when incubated at pH 4.6 or pH 5.1 (Fig. 16). Incubation at pH 5.8-7.4 did not yield any significant association of liposomes with cells. These results suggest that these liposomes are sensitive to elastase-mediated activation of binding and lipid mixing when DODAP is maximally positively charged. The pH required to achieve this state is present under normal physiological conditions with the late endosome (Kielian et al., 1986).

To confirm that the enzymatic activity of elastase was responsible for triggering of binding and lipid mixing, DODAP/MeO-suc-AAPV-DOPE liposomes were pretreated with heat inactivated elastase. Heating elastase to 95 degrees C for 1 hour completely abrogated enzymatic cleavage of either a chromogenic substrate or MeO-suc-AAPV-DOPE. Pretreatment of DODAP/MeO-suc-AAPV-DOPE liposomes with heat inactivated elastase did not enhance binding or lipid mixing with HL60 cells above background levels (Fig. 17). Only active elastase was capable of triggering this increased association. To observe this effect, pH 5 conditions were required, since at pH 7.4 the binding of these liposomes to HL60 cells was greatly reduced (Figs. 17A and 17B).

Example 17

Fluorescence microscopy of liposome-cell lipid mixing with HL60 cells

DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were incubated for 2 hours at 37°C without or with elastase (5 • g protein/100 nmol lipid). Liposomes containing the fluorescent lipid probes *N*-NBD-PE and *N*-Rho-PE were bound to HL60 cells in solution as described above. Cells were washed with HBSS to remove unbound liposomes and observed with a Bio-Rad (Hercules, CA) MRC-1000 laser scanning confocal imaging

system equipped with an Olympus BX50 microscope (Olympus Corp., Lake Success, NY) using an apochromat 60x oil (N.A. 1.40) objective. *N*-Rho-PE fluorescence was observed with the 568 nm line and observed at 605 nm emission. Images were frame averaged and false color was applied. All images within a figure were obtained under identical conditions of confocal iris width, gain, and black level. Identical false color look up tables were also applied to images within a figure. Average fluorescence/cell μm^2 of all cells in an image was determined with the histogram feature of the Bio-Rad CoMOS confocal imaging software.

Confocal microscopy of liposomes mixed with HL60 cells confirmed that elastase pretreatment was required for enhanced binding and lipid mixing. Brightly fluorescently labeled cells indicate the *N*-Rho-PE fluorescent probe from the bound elastase-activated liposomes had mixed into the cell plasma membrane (Fig. 18). In contrast, untreated liposomes displayed significantly less binding and lipid mixing (Fig. 18). Quantitation of fluorescence images revealed nearly 12 times as much fluorescence/cell area in HL60 cells that had been incubated with DODAP/MeO-suc-AAPV-DOPE liposomes treated with elastase, as compared to those cells incubated with untreated liposomes, although the distribution of this enhanced delivery may not be uniform across all the cells. Almost no fluorescence was associated with the cells at pH 7.4.

Example 18

Preparation of fluorescent dextran-containing liposomes and calcein-loaded liposomes

Fluorescent dextran containing liposomes were prepared by hydrating the DODAP/MeO-suc-AAPV-DOPE lipid film described previously (see Example 5) with a 50 mg/ml solution of 10,000 MW tetramethyl rhodamine dextran (TMR-dextran) in TES/NaCl/EDTA buffer. Liposomes were then vortexed, freeze/thawed, and extruded through 0.1 μm filters as described above. To remove unencapsulated dextran the liposome solution was extensively dialyzed with TES/NaCl/EDTA buffer using a Biodialyser (Sialomed, Columbia, MD) fitted with 50 nm pore size filters. Calcein-loaded liposomes

were prepared by hydrating the lipid film in the presence of buffer containing 50mM calcein. Calcein (> 95% pure) and tetramethylrhodamine dextran were obtained from Molecular Probes (Eugene, OR). Calcein solution was adjusted to a pH of approximately 7 and an osmolarity of approximately 300 mosm prior to preparation of liposomes. Calcein-loaded liposomes contained 0.75 mol % *N*-Rho-PE to monitor liposome binding. After preparation of large vesicles as described above, calcein-loaded liposomes were transferred to a 10,000 molecular weight cut off (MWCO) Slide-A-Lyzer (Pierce, Rockford, IL) and extensively dialyzed with TES/NaCl/EDTA buffer. The encapsulated volume of these liposomes was 0.8 l/mol of lipid. Sonicated vesicles were prepared by drying lipid in the same manner as described above but preparations were vortexed then water bath sonicated for >10 min at room temperature. Lipid concentration was monitored by phosphate assay (Bartlett 1959). The size of liposomes was verified by quasi-elastic light scattering using a Nicomp Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Freeze-thaw/extrusion vesicles and sonicated vesicles were 70-80 nm and 35-45 nm in diameter, respectively, as determined by number weighted Gaussian analysis.

Example 19

Aqueous contents delivery from DODAP/MeO-suc-AAPV-DOPE liposomes to HL60 cells

To determine if the enhanced lipid mixing between elastase-activated DODAP/MeO-suc-AAPV-DOPE liposomes and HL60 cells is truly indicative of fusion, the delivery of an aqueous probe from the liposome to the cell cytoplasm was monitored DODAP/MeO-suc-AAPV-DOPE liposomes were loaded with tetramethyl rhodamine labeled 10,000 MW dextran (TMR-dextran), treated with or without elastase, and incubated with HL60 cells under pH 5 conditions.

TMR-dextran loaded DODAP/MeO-suc-AAPV-DOPE liposomes (40 nmol, prepared as described in Examples 5 and 16) were incubated with $1x10^5$ HL60 cells in 200 • l TES/NaCl/EDTA buffer under pH 5, 37°C, conditions for 30 min to induce binding. TMR-dextran fluorescence was observed by confocal microscopy under the same conditions as N-Rho-PE described in Example 17.

Only DODAP/MeO-suc-AAPV-DOPE liposomes that had been pretreated with elastase were capable of fusing with HL60 cells, as demonstrated by TMR-dextran labeling of the cytoplasm of these cells (Fig. 19). HL60 cells incubated with liposomes that had not been treated with elastase contained little or no cytoplasmic fluorescent dextran, indicating elastase cleavage was required to trigger the fusion of DODAP/MeO-suc-AAPV-DOPE liposomes with HL60 cells.

TMR-dextran delivery to cells was not due to leakage of the fluorescent dextran out of the liposomes and subsequent uptake by HL60 cells. This possibility was investigated by inducing TMR-dextran release from DODAP/MeO-suc-AAPV-DOPE liposomes. The results demonstrated that there was no uptake into the HL60 cells.

Example 20

DODAP/MeO-suc-AAPV-DOPE liposome interaction with EVC304 cells:binding, lipid mixing and calcein delivery

Liposomes were bound to adherent ECV304 cells via a biotin-streptavidin linkage. To this end DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) liposomes were prepared with 0.3 mol% *N*-biotinyl caproylamine-PE (N-biotinyl-cap-PE) as well as fluorescent lipid probes or with encapsulated calcein. ECV304 cells that had been plated on glass coverslips in tissue culture plates were washed with HBSS buffer and then incubated sequentially at room temperature with biotin-wheat germ agglutinin (WGA) (20 • g/ml, obtained from Pierce, Rockford, IL) and streptavidin (40 • g/ml, obtained from Molecular Probes, Eugene, OR) prepared in HBSS, 30 min/treatment. Cells were washed after each treatment. Liposomes were treated with or without elastase as described above. Certain aliquots of pretreated DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were freeze/thawed after dialysis and prior to addition to cells to release the liposomal contents. Such freeze/thawed liposomes were exposed to liquid nitrogen/37°C water bath for 5 cycles. Self-quenching of calcein was reduced by approximately 85% (maximal FDQ determined by detergent solubilization) after freeze/thawing, indicating release of encapsulated calcein. In all cases, 50-100 nmol of liposomes were added to confluent ECV304 cell monolayers ($1x10^5$ cells/well of 24 well

plate) and incubated in HBSS for 30 min at room temperature to promote *N*-biotinyl cap-PE binding to streptavidin. Unbound liposomes were removed by repeated washes. After the final wash, fresh HBSS buffer was added to all wells and cells were incubated at 37°C for given times. Fluorescence was quantitated as described above. Calcein fluorescence was excited with the 488 nm line of a krypton/argon laser and observed at 522 nm emission

Example 21

Elastase-activated binding and, lipid mixing of DODAP/MeO-suc-AAPV-DOPE liposomes with adherent ECV304 cells

To determine whether elastase-treated liposomes internalized within mammalian cells by endocytosis could be activated to bind and fuse with the cells, liposomes were bound to cells under physiological conditions at pH 7.4 via a biotin-streptavidin linkage. The pretreatment of DODAP/MeO-suc-AAPV-DOPE liposomes with elastase activated these liposomes to bind and fuse with HL60 cells when the pH was artificially lowered to mimic the endosomal environment (Fig. 19). The adherent cell line, ECV304 was selected to circumvent complications arising from biotin-streptavidin mediated aggregation of the suspension HL60 cells.

ECV304 cells were sequentially treated with biotinylated-wheat germ agglutinin and streptavidin. DODAP/MeO-suc-AAPV-DOPE liposomes containing trace amounts of *N*-biotinyl cap-PE and pretreated with or without elastase were then added to the cells and incubated at pH 7.4. Elastase pretreated liposomes exhibited enhanced binding, as compared to untreated liposomes (Fig. 20), perhaps as a result of decreased negative surface charge or better accessibility of the biotinyl group. It appears that elastase activation augments the biotin-streptavidin mediated binding. The biotin-streptavidin linkage was required, as the absence of biotin-streptavidin treatment abrogated any binding of untreated or elastase pretreated liposomes at this pH (unpublished data). Liposomes with or without elastase activation were localized within perinuclear endocytic vesicles (Fig. 21). There was enhanced binding of elastase pretreated liposomes and uptake into endocytic vesicles; however, there was only a slight increase in lipid mixing of these liposomes with ECV304 cells (Fig. 20). This may be due to the nature of the assay because the fluorescence dequenching assay requires the diffusion of the *N*-

NBD-PE and *N*-Rho-PE probe after lipid mixing. Diffusion of the fluorescent lipid probes after fusion with the endosomal membrane may be insufficient to completely diminish the resonance energy transfer. Therefore an aqueous contents delivery assay was employed.

Example 22

Aqueous contents delivery of calcein from DODAP/MeO-suc-AAPV-DOPE liposomes to adherent ECV304 cells

DODAP/MeO-suc-AAPV-DOPE liposomes were loaded with self-quenched concentrations of calcein, a fluorescent aqueous probe, as previously described (Examples 5 and 18). After extensive dialysis to remove unencapsulated calcein these liposomes were treated with or without elastase and bound to ECV304 cells by biotinylated WGA-streptavidin. Elastase pretreated liposomes displayed relatively rapid calcein dequenching that increased over the course of several hours (Fig. 22). Maximal dequenching of calcein appears to be achieved after 2 hours at 37°C, which was consistent with the time course of DODAP/MeO-suc-AAPV-DOPE liposome endocytosis (unpublished data) and cationic lipid:DNA complex uptake [Zabner et al., 1995]. The apparent delivery of calcein was completely dependent upon liposome-cell interactions, as calcein-loaded liposomes subjected to identical conditions of elastase pretreatment and pH 5 environment but without co-incubation with cells did not demonstrate any increase in calcein fluorescence dequenching. DODAP/MeO-suc-AAPV-DOPE liposomes that had not been treated with elastase exhibited much less initial calcein dequenching that slowly increased over time, albeit not to levels observed with the elastase-activated liposomes (Fig. 22). Repeated freeze/thaw cycles of DODAP/MeO-suc-AAPV-DOPE liposomes results in almost complete release of encapsulated calcein. Incubation of this mixture of freeze/thawed liposomes and released free calcein with ECV304 cells resulted in only 2-3% of the calcein uptake observed with cells incubated with intact liposomes. Thus the fluorescence dequenching observed with elastase treated intact liposomes was due to delivery of calcein to the cells rather than a non-specific uptake of free calcein. The results with the adherent endothelial cell line ECV304 are similar to those obtained with the HL60 leukemia cell line described in earlier examples.

Confocal microscopy of ECV304 cells incubated with elastase pretreated DODAP/MeO-suc-AAPV-DOPE liposomes contained brightly fluorescent perinuclear localized vesicles that appear to be due to uptake of the calcein-loaded liposomes into endosomes. Importantly, these cells also had diffuse fluorescence within the cells (Fig. 21) that is indicative of calcein delivery into the cytosol. The diffuse fluorescence was not due to non-specific uptake of calcein released from liposomes prior to endocytosis, as loaded liposomes subjected to freeze/thaw cycles did not result in fluorescently labeled cells. Unactivated liposomes were also endocytosed into vesicles near the nucleus. However, there was no diffuse fluorescence visible with these liposomes. Quantitation of total fluorescence/cell area showed ECV304 cells incubated with elastase-activated DODAP/MeO-suc-AAPV-DOPE liposomes had more than twice the amount of calcein fluorescence as those incubated with unactivated liposomes. Without elastase pretreatment there does not appear to be significant delivery of the encapsulated calcein into the cell cytoplasm.

Example 23

Abbreviations

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
DCC	Dicyclohexyl carbodiimide
DC-Chol	3-beta-[N-[(N'.N'-dimethylamino)ethane]carbamoyl]cholesterol
DCU	Dicyclohexyl urea
DMRI	1,2-dimyristtooxypropyl-3-dimethylhydroxyethyl ammonium bromide
DODAP	1-N,N-drimethylamino dioleoyl propane
DOPE	Dioloeoyl phosphatidylethanolamine
DORIE	1,2-dioleooxypropyl-3-dimethylhydroxyethyl ammonium bromide
DOTAP	1,2-bis(oleoyloxy)-3-trimethylammonio)propane
DPPE	Dipalmityl phosphatidylethanolamine
DSPE	Distearoylphosphatidylethanolamine
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)-tetraacetic acid
FBS	Fetal bovine serum
FDQ	Fluorescence dequenching
HBSS	Hanks' Balanced Salts solution
HLE	Human leukocyte elastase
LUV	Large Unilamellar vesicles
MeO-suc	Methoxy succinyll
MLV	Multilamellar vesicles
N-biotinyl-cap-PE	N-biotinyl caproylamine-PE
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
POPE	Palmitoyloleoyl phosphatidylethanolamine
PS	Phosphatidylserine
RBC	Red blood cell
SUV	Small unilamellar vesicles
TFA	Trifluoracetic acid

THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMR-dextran	Tetramethyl rhodamine dextran
tPE	Transesterified phosphatidylethanolamine
WGA	Wheat germ agglutinin.

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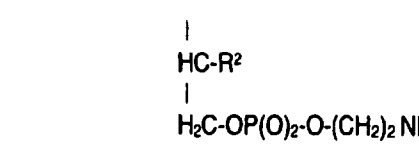
TLC-215C

What is claimed is:

1. A method of administering the contents of a liposome to a mammal which comprises administering to the mammal a composition comprising:

(i) a pharmaceutically acceptable carrier; and,

(ii) a liposome comprising a lipid component which comprises a peptide-lipid conjugate having the formula:



wherein:

X is a linker selected from the group consisting of a single bond or the group R^3;

each of R^1 and R^2 is

$-OC(O)(CH_2)_{n1}(CH=CH)_{n2}(CH_2)_{n3}(CH=CH)_{n4}(CH_2)_{n5}(CH=CH)_{n6}(CH_2)_{n7}(CH=CH)_{n8}(CH_2)_{n9}CH_3$; and

R^3 is

$-C(O)(CH_2)_{n1}(CH=CH)_{n2}(CH_2)_{n3}(CH=CH)_{n4}(CH_2)_{n5}(CH=CH)_{n6}(CH_2)_{n7}(CH=CH)_{n8}(CH_2)_{n9}HN-$.

n1 is zero or is an integer equal to from 1 to 22,

n3 is zero or is an integer equal to from 1 to 19,

n5 is zero or is an integer equal to from 1 to 16,

n7 is zero or is an integer equal to from 1 to 13, and

n9 is zero or is an integer equal to from 1 to 10;

for each of R^1 and R^2 the sum of n1 + 2n2 + n3 + 2n4 + n5 + 2n6 + n7 + 2n8 + n9 is an integer equal to from 12 to 22;

for R^3 the sum of n1 + 2n2 + n3 + 2n4 + n5 + 2n6 + n7 + 2n8 + n9 is zero or is an integer equal to from 1 to 22;

each of n2, n4, n6 and n8 is equal to 0 or 1;

Y is a peptide comprising an amino acid sequence which is the substrate of a cell-secreted or cell-associated peptidase; and

the contents of the liposome are delivered to the vicinity of cells in the mammal which secrete a peptidase which recognizes the amino acid substrate.

2. The method of claim 1, wherein the liposome is a large unilamellar liposome.

3. The method of claim 1, wherein the peptide comprises the amino acid sequence Ala-Ala.

4. The method of claim 3, wherein the peptide comprises an amino acid sequence selected from the group consisting of the sequences Ala-Ala, Ala-Ala-Pro-Val (SEQ ID NO: 1), Ala-Ala-Met-, Ala-Ala-Pro-Phe (SEQ ID NO: 3), Ala-Ala-Pro-Met (SEQ ID NO: 4), Ala-Ala-Arg, Ser-Ala-Ala-Arg (SEQ ID NO: 5), Ser-Ser-Ala-Ala-Arg (SEQ ID NO: 6), Ser-S carboxyl sugar-Ala-Ala-Arg- (SEQ ID NO: 7), Ala-Ala-Asp-, Ser-Ala-Ala-Asp- (SEQ ID NO: 8) and Ser-Ser-Ala-Ala-Asp- (SEQ ID NO: 9).

5. The method of claim 4, wherein the peptide comprises the amino acid sequence Ala-Ala-Pro-Val (SEQ ID NO: 1).

6. The method of claim 1, wherein the peptide comprises an amino acid sequence selected from the group consisting of Arg-Pro-Lys-Pro-Leu-Ala-Nva- (SEQ ID NO: 10), Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva- (SEQ ID NO: 11), Ser-Ser-Arg-Pro-Lys-Pro-Leu-Ala-Nva- (SEQ ID NO: 12), Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 (SEQ ID NO: 13), Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (SEQ ID NO: 14), ; Pro-Cha-Gly-Nva-; Pro-Leu-Gly-Leu- (SEQ ID NO: 15) and Gly-Pro-Gln-Gly-Ile- (SEQ ID NO: 16).

7. The method of claim 1, wherein the peptide is modified at its amino terminus by a moiety selected from the group consisting of acetyl, methoxy, carboxy sugar, polyethylene glycol and methoxy-substituted carboxy sugar modifications.

8. The method of claim 7, wherein the peptide is preferably N-acetyl modified at its amino terminus and the peptide-lipid conjugate comprises from about 20 mole % to about 80 mole % of the lipid component.

9. The method of claim 1, wherein X is a single bond, each of R^1 and R^2 is $-OC(O)(CH_2)_7(CH=CH)(CH_2)_7CH_3$ and the peptide comprises the amino acid sequence Ala-Ala.

10. The method of claim 9, wherein the peptide-lipid conjugate is:

H_2C-$OC(O)(CH_2)_7(CH=CH)(CH_2)_7CH_3$
|
HC-$OC(O)(CH_2)_7(CH=CH)(CH_2)_7CH_3$
|
H_2C-$OP(O)_2$-O-$(CH_2)_2$–NH -Val-Pro-Ala-Ala;

the peptide is N-methoxysuccinyl modified at its amino terminus and the peptide-lipid conjugate comprises from about 20 mole % to about 80 mole % of the lipid component.

11. The method of claim 10, wherein the lipid component further comprises an additional lipid which is a positively charged lipid selected from the group consisting of 1,2-bis(oleoyloxy)-3(trimethylammonio)propane (DOTAP); 1-N,N-dimethylamino dioleoyl propane (DODAP); 1-oleoyl- 2-hydroxy-3-N,N-dimethylamino propane; 1,2-diacyl-3-N,N-dimethylamino propane; 1,2-didecanoyl -1-N,N,-dimethylamino propane, 3-beta-[N-[(N',N'-dimethylamino)ethane]carbamoyl]cholesterol (DC-Chol), 1,2-dimyristooxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE); and 1,2-dioloeooxypropyl-3-dimethylhydroxyethyl ammonium bromide (DORI).

12. The method of claim 11, wherein the positively charged lipid is DODAP.

13. The method of claim 12, wherein the lipid component comprises 50 mole % DODAP and 50 mole % of the peptide-lipid conjugate.

14. The method of claim 12 , wherein the lipid component comprises DODAP and N-Ala-Ala-Pro-Val-DOPE.

15. The method of claim 11, wherein the positively charged lipid is DOTAP.

16. The method of claim 15, wherein the lipid component comprises 50 mole % DOTAP and 50 mole % of the peptide-lipid conjugate.

17. The method of claim 15, wherein the lipid component comprises 50 mole % DOTAP and 50 mole % N-Ala-Ala-Pro-Val.

18. The method of claim 10, wherein the lipid component further comprises an additional lipid selected from the group consisting of trans-esterified phosphatidylethanolamine (tPE), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE) and dioleoyl phosphatidylethanolamine (DOPE).

19. The method of claim 1, wherein the peptidase is selected from the group consisting of elastase, plasmin, plasminogen activator, urokinase; stromelysin, human collagenases, cathepsins, lysozyme, granzymes, dipeptidyl peptidases, peptide hormone-inactivating enzymes, kininases, bacterial peptidases and viral proteases.

20. The method of claim 19, wherein the peptidase is elastase.

21. The method of claim 19, wherein the peptidase is stromelysin, a cathepsin, plasmin or a plasminogen activator.

22. The method of claim 1, wherein the liposome further comprises a bioactive agent selected from the group consisting of antiviral agents, antibacterial agents, antifungal agents, antineoplastic agents, antiinflammatory agents, radiolabels, radiopaque compounds, fluorescent compounds, mydriatic compounds, bronchodilators, local anesthetics, nucleic acid sequences, plasmid deoxyribonucleic acid sequences and bioactive lipids.

23. The method of claim 22, wherein the bioactive agent is an anticancer agent, the liposome contains a therapeutically effective amount of the anticancer agent and the mammal is afflicted with a cancer.

24. The method of claim 23, wherein the cancer is selected from the group consisting of brain cancers, breast cancers, carcinomas, colon cancers, leukemias, lung cancers, lymphomas, ovarian cancers and sarcomas.

25. The method of claim 22, wherein the bioactive agent is an anti-inflammatory agent, the liposome comprises a therapeutically effective amount of the anti-inflammatory agent and the mammal is afflicted with an inflammatory disorder.

26. The method of claim 22, wherein the bioactive agent is a nucleic acid, the mammal is afflicted with a genetic disorder and the nucleic acid encodes a protein which ameliorates the disorder.

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Arg Pro Lys Pro Leu Ala Xaa
1 5

<210> 24
<211> 7
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal methoxy succinyl group

<221> MOD_RES
<222> (7)...(7)
<223> Nva

<400> 24
Arg Pro Lys Pro Leu Ala Xaa
1 5

<210> 25
<211> 7
<212> PRT

<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal carboxy sugar group

<221> MOD_RES
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<223> Nva

<400> 25
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1 5

<210> 26
<211> 7
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal succinyl group

<221> MOD_RES
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<223> Nva

<400> 26
Arg Pro Lys Pro Leu Ala Xaa
1 5

<210> 27
<211> 8
<212> PRT
<213> Peptides

<220>
<221> BINDING
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<223> N-terminal acetyl group

<221> MOD_RES
<222> (8)...(8)
<223> Nva

<400> 27
Ser Arg Pro Lys Pro Leu Ala Xaa
1 5

<210> 28
<211> 8
<212> PRT
<213> Peptides

<220>

<221> BINDING
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<223> N-terminal methoxy succinyl group

<221> MOD_RES
<222> (8)...(8)
<223> Nva

<400> 28
Ser Arg Pro Lys Pro Leu Ala Xaa
1 5

<210> 29
<211> 9
<212> PRT
<213> Peptides

<220>
<221> BINDING
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<223> N-terminal acetyl group

<221> MOD_RES
<222> (9)...(9)
<223> Nva

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1 5

<210> 30
<211> 9
<212> PRT
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<220>
<221> BINDING
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<223> N-terminal methoxy succinyl group

<221> MOD_RES
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<223> Nva

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1 5

<210> 31
<211> 4
<212> PRT
<213> Peptides

<220>
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<400> 31
Ala Ala Pro Phe
1

<210> 32
<211> 4
<212> PRT
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<220>
<221> BINDING
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<400> 32
Ala Ala Pro Met
1

<210> 33
<211> 4
<212> PRT
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<220>
<221> BINDING
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<400> 33
Ala Ala Pro Met
1

<210> 34
<211> 4
<212> PRT
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<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal acetyl group

<400> 34
Ser Ala Ala Arg
1

<210> 35
<211> 4
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<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal methoxy succinyl group

<400> 35
Ser Ala Ala Arg
1

<210> 36
<211> 5
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)
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<400> 36
Ser Ser Ala Ala Arg
1 5

<210> 37
<211> 5
<212> PRT
<213> Peptides

<220>
<221> BINDING
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<400> 37
Ser Ser Ala Ala Arg
1 5

<210> 38
<211> 4
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<221> BINDING
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<400> 38
Ser Ala Ala Asp
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<210> 39
<211> 4
<212> PRT
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<220>
<221> BINDING
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<400> 39

Ser Ala Ala Asp
1

<210> 40
<211> 5
<212> PRT
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<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal acetyl group

<400> 40
Ser Ser Ala Ala Asp
1 5

<210> 41
<211> 5
<212> PRT
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<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal methoxy succinyl group

<400> 41
Ser Ser Ala Ala Asp
1 5

<210> 42
<211> 4
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)
<223> N-terminal N-methoxysuccinyl group

<221> BINDING
<222> (4)...(4)
<223> C-terminal dioleoyl phosphatidylethanolamine

<400> 42
Ala Ala Pro Val
1

<210> 43
<211> 4
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (1)...(1)

<223> N-terminal N-methoxysuccinyl group

<221> BINDING
<222> (4)...(4)
<223> C-terminal phosphatidylethanolamine

<400> 43
Ala Ala Pro Val
1

<210> 44
<211> 4
<212> PRT
<213> Peptides

<220>
<221> BINDING
<222> (4)...(4)
<223> C-terminal dioleoyl phosphatidylethanolamine

<400> 44
Ala Ala Pro Val
1

Fig. 1

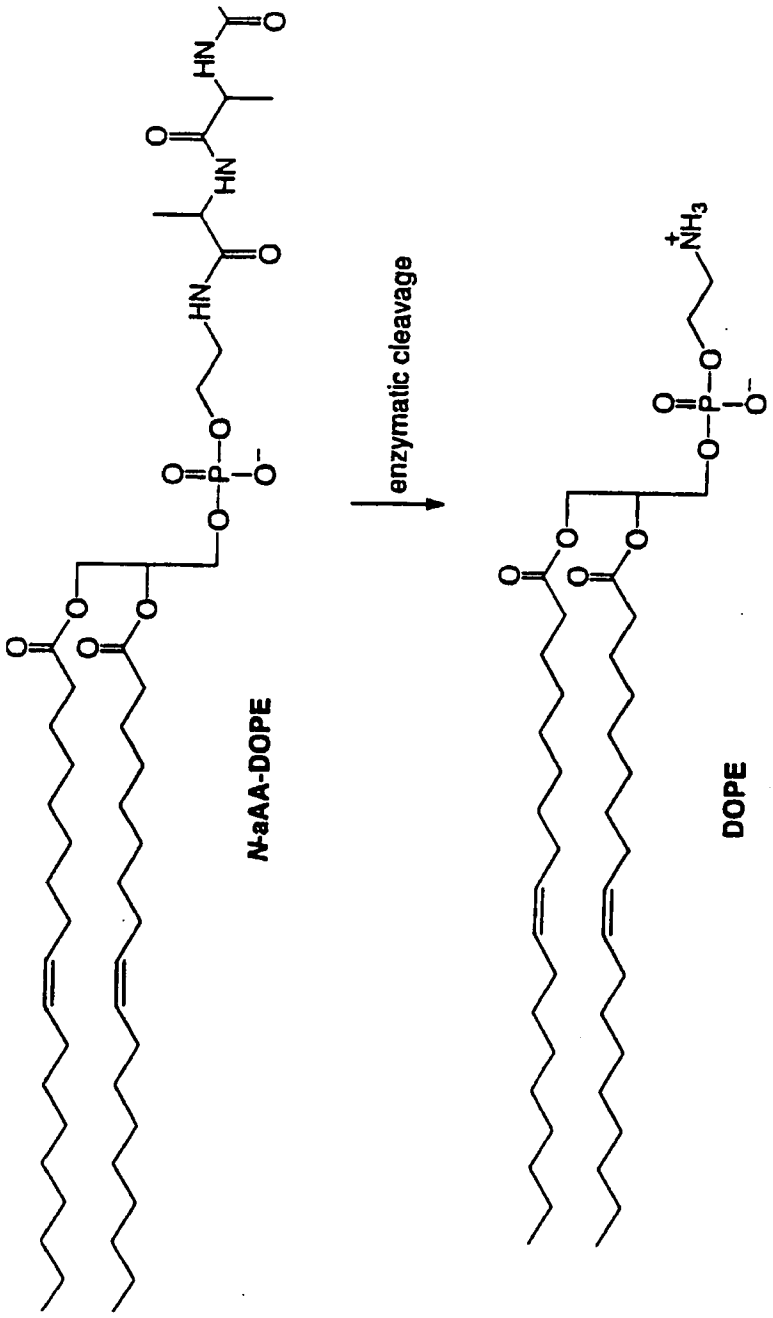
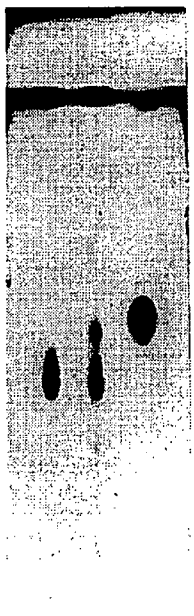
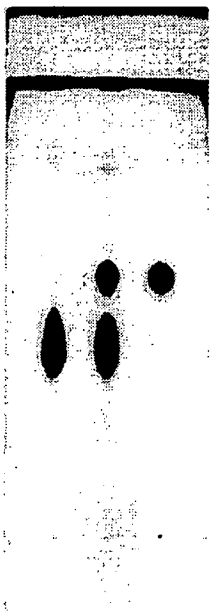


Fig. 2
A



1 2 3

Fig. 2
B



1 2 3

Fig. 3

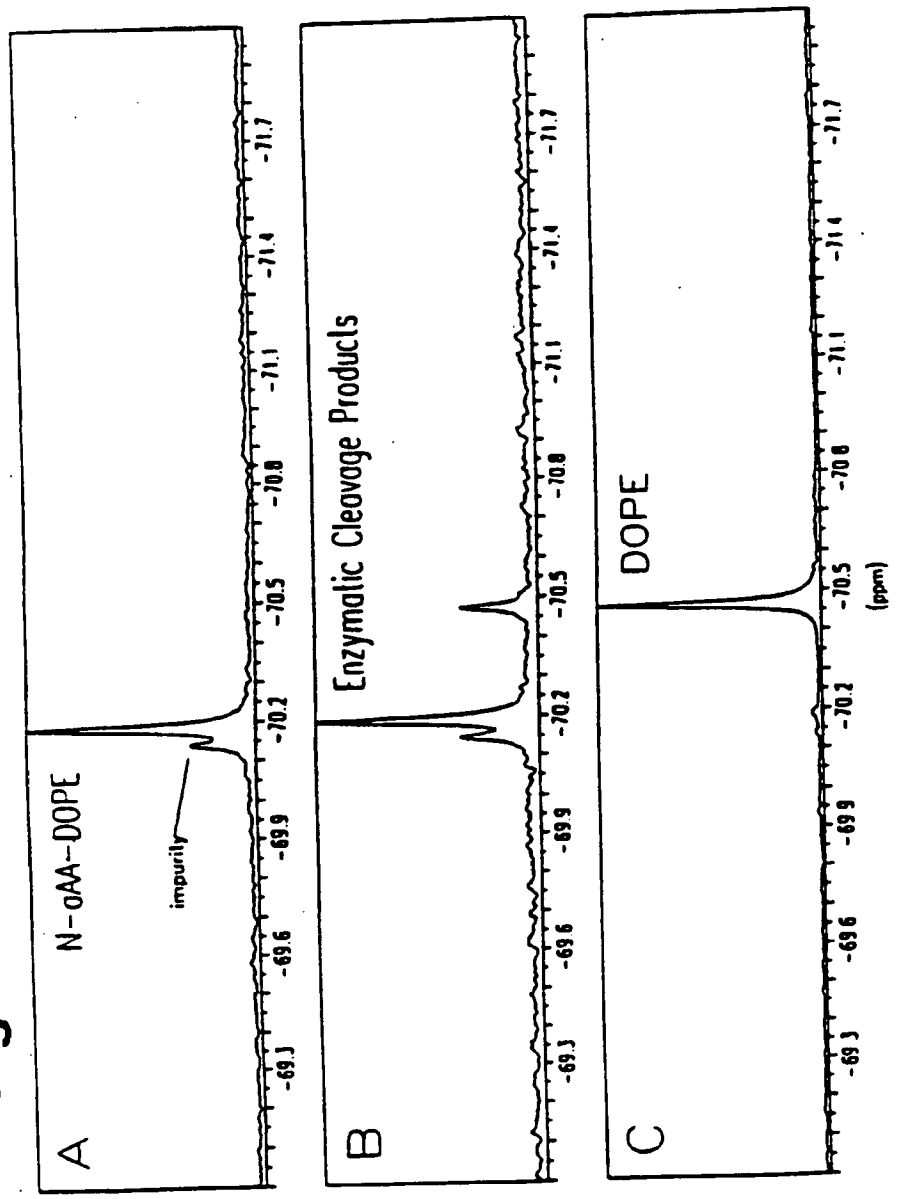

A
N-oAA-DOPE
impurity
B
Enzymatic Cleavage Products
C
DOPE
-69.3
-69.6
-69.9
-70.2
-70.5
-70.8
-71.1
-71.4
-71.7
(ppm)

Fig. 4

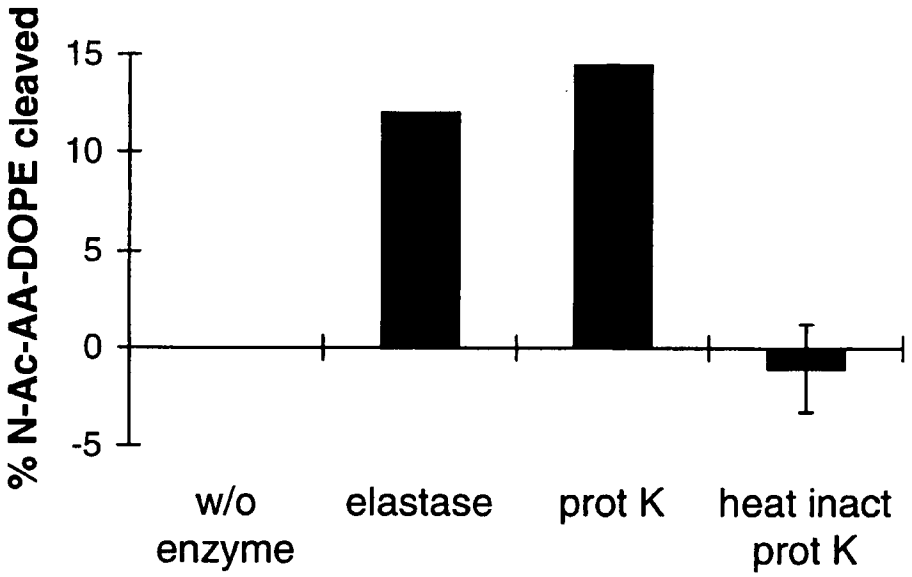
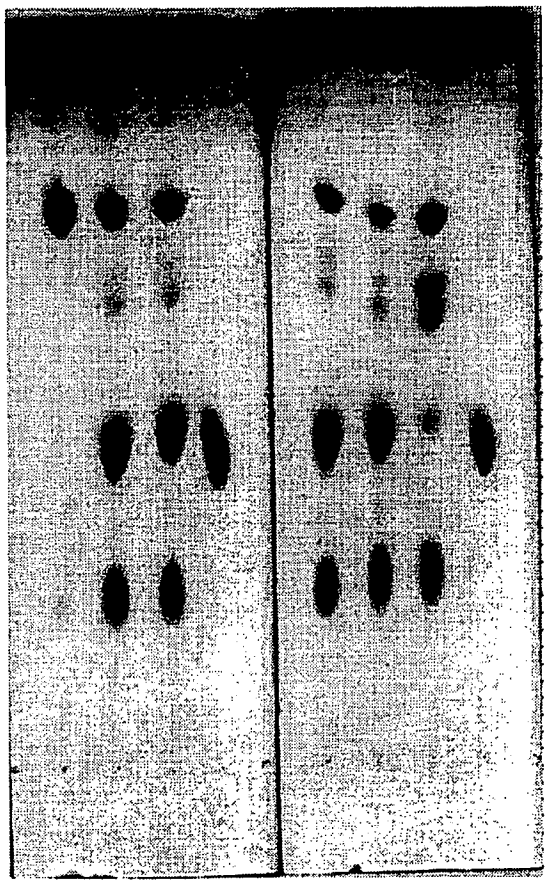

% N-Ac-AA-DOPE cleaved
15
10
5
0
-5
w/o enzyme
elastase
prot K
heat inact prot K

Fig. 5A



1 2 3 4 5 6 7 8

Fig. 5B

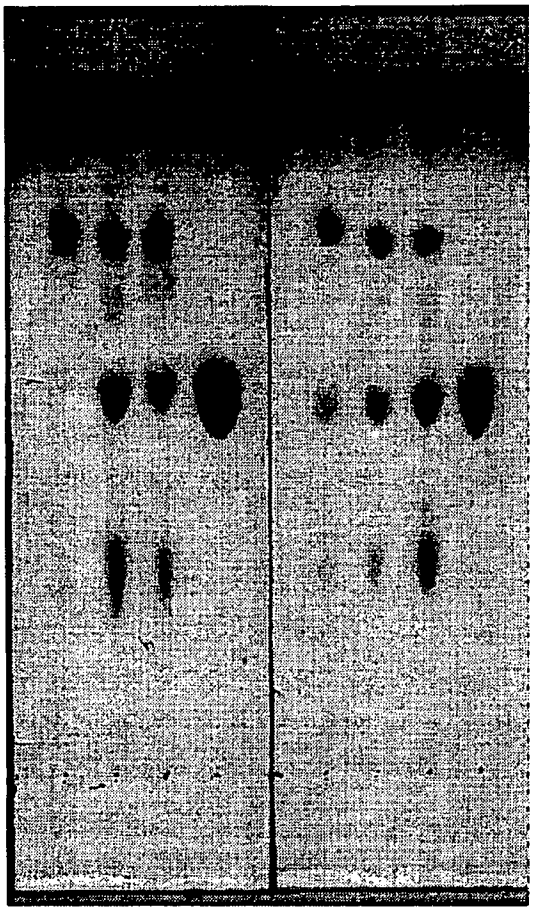


Fig. 5C

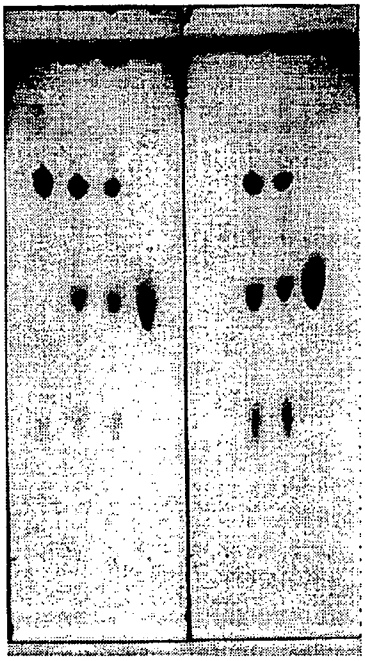


Fig. 6

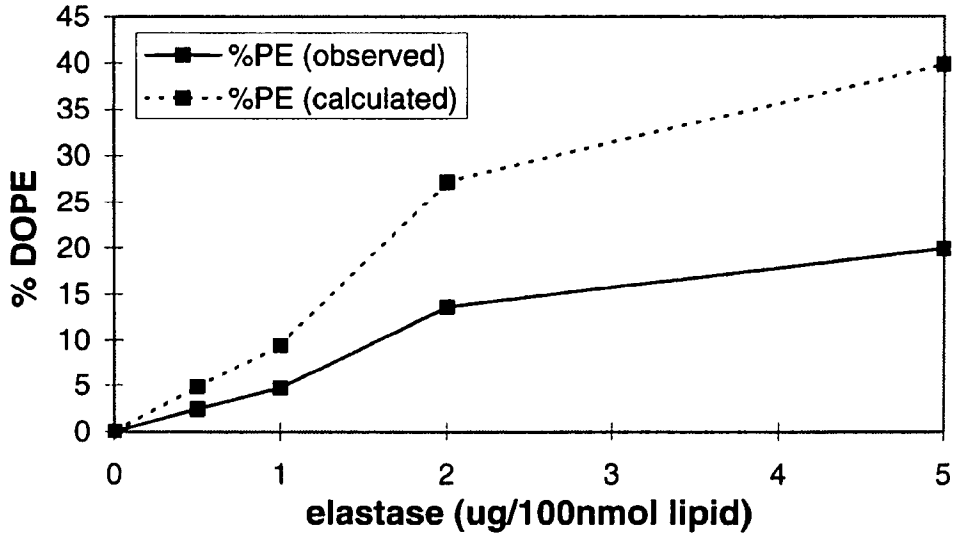

45
40
35
30
25
20
15
10
5
0
% DOPE
%PE (observed)
%PE (calculated)
0
1
2
3
4
5
elastase (ug/100nmol lipid)

Fig. 7

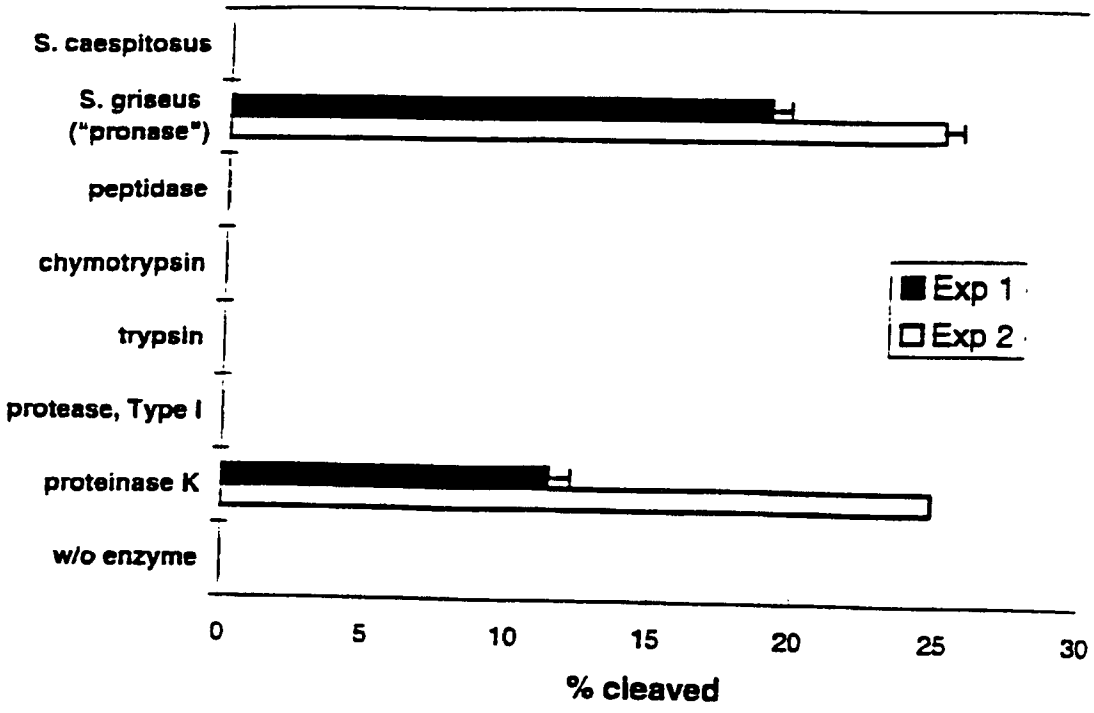

S. caespitosus
S. griseus ("pronase")
peptidase
chymotrypsin
trypsin
protease, Type I
proteinase K
w/o enzyme
Exp 1
Exp 2
0
5
10
15
20
25
30
% cleaved

Fig. 8A

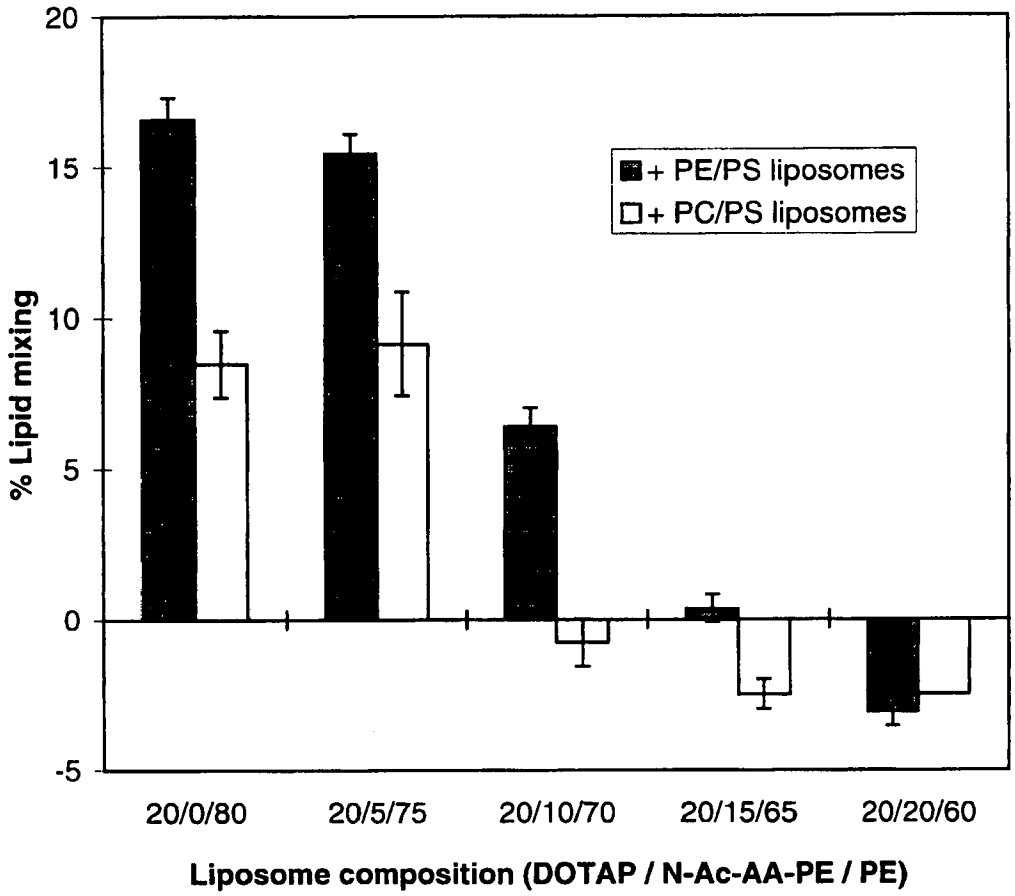

20
15
10
5
0
-5
% Lipid mixing
+ PE/PS liposomes
+ PC/PS liposomes
20/0/80
20/5/75
20/10/70
20/15/65
20/20/60
Liposome composition (DOTAP / N-Ac-AA-PE / PE)

Fig. 8B

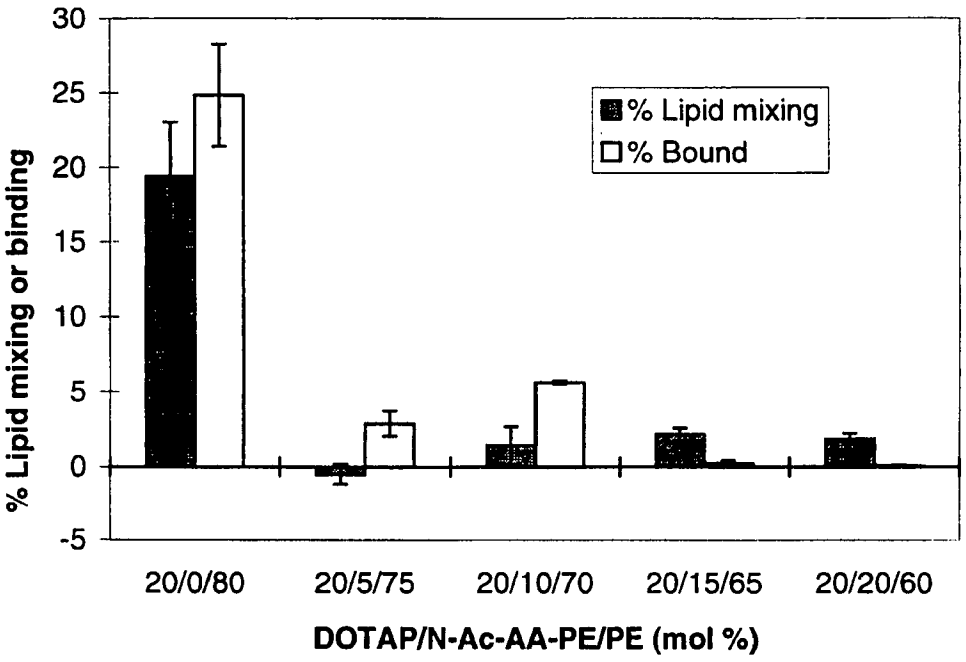

30
25
20
15
10
5
0
-5
% Lipid mixing or binding
% Lipid mixing
% Bound
20/0/80
20/5/75
20/10/70
20/15/65
20/20/60
DOTAP/N-Ac-AA-PE/PE (mol %)

Fig. 9A

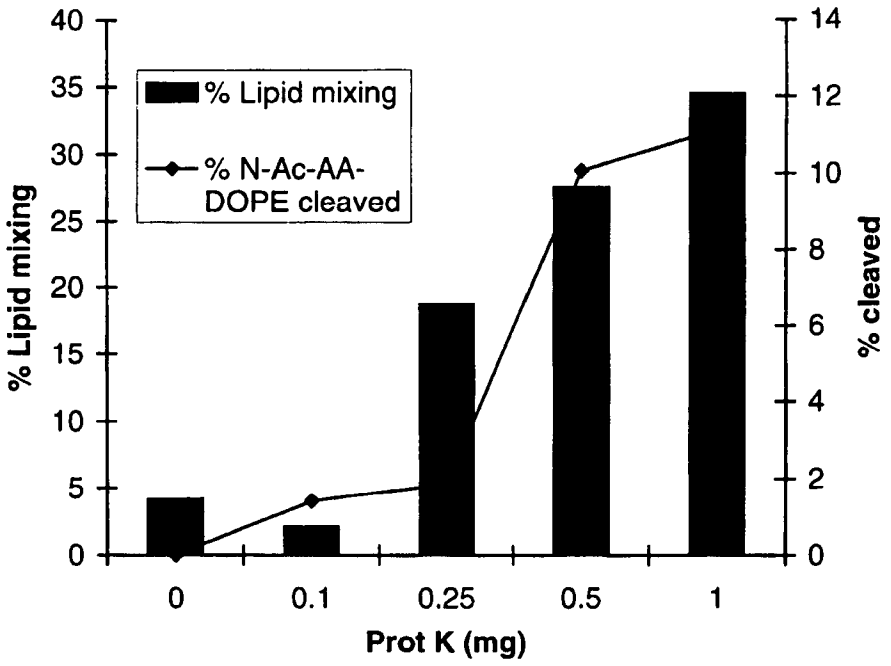

40
35
30
25
20
15
10
5
0
% Lipid mixing
% Lipid mixing
% N-Ac-AA-DOPE cleaved
14
12
10
8
6
4
2
0
% cleaved
0
0.1
0.25
0.5
1
Prot K (mg)

Fig. 9B

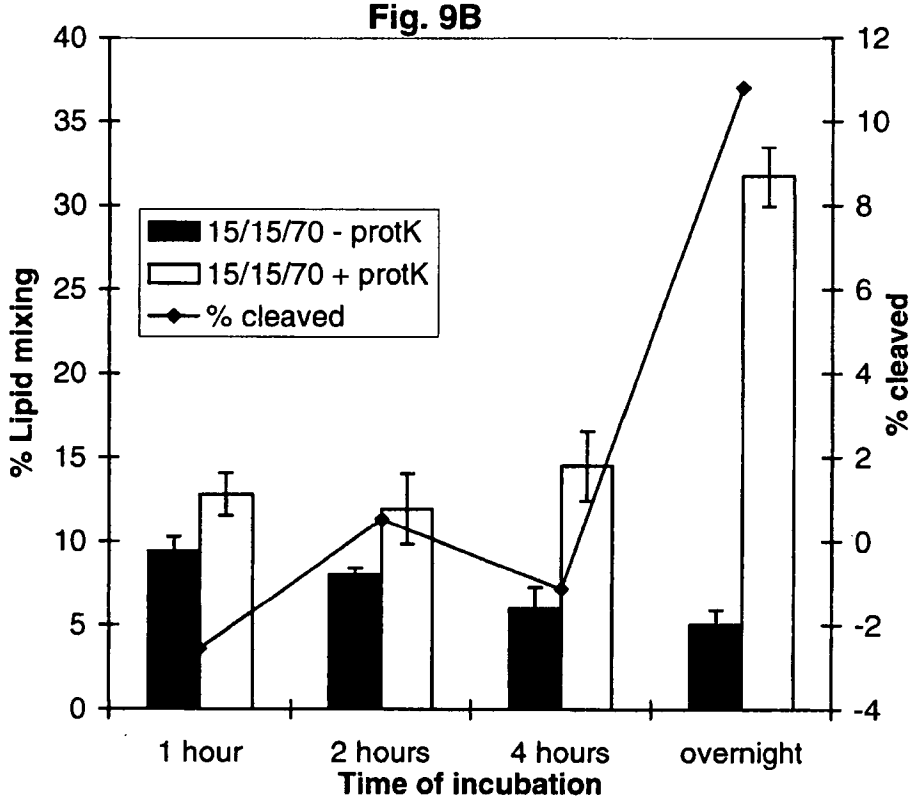

40
35
30
25
20
15
10
5
0
12
10
8
6
4
2
0
-2
-4
% Lipid mixing
% cleaved
15/15/70 - protK
15/15/70 + protK
% cleaved
1 hour
2 hours
4 hours
overnight
Time of incubation

Fig. 10

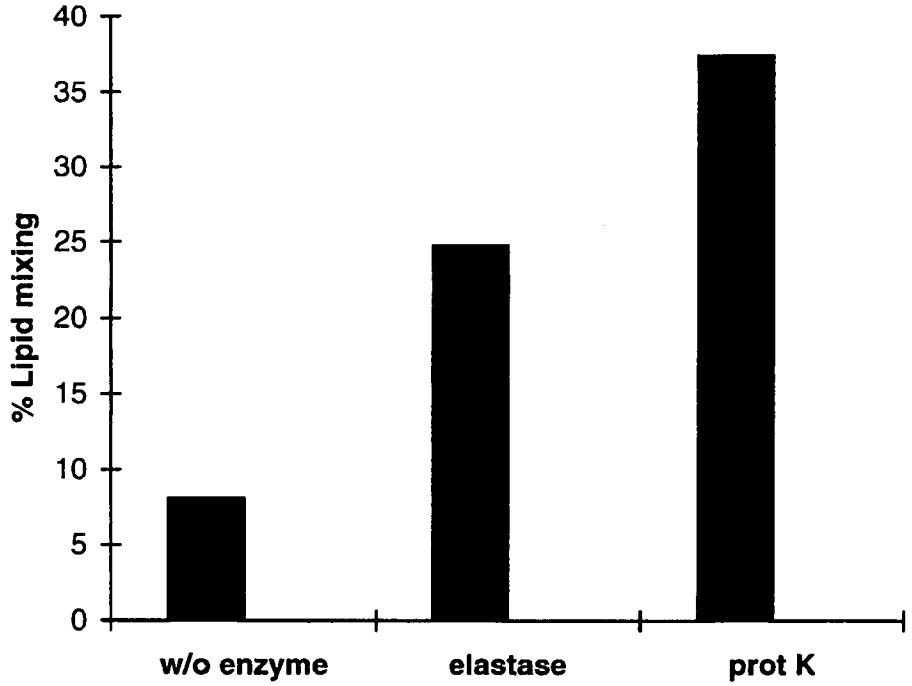


Fig. 11

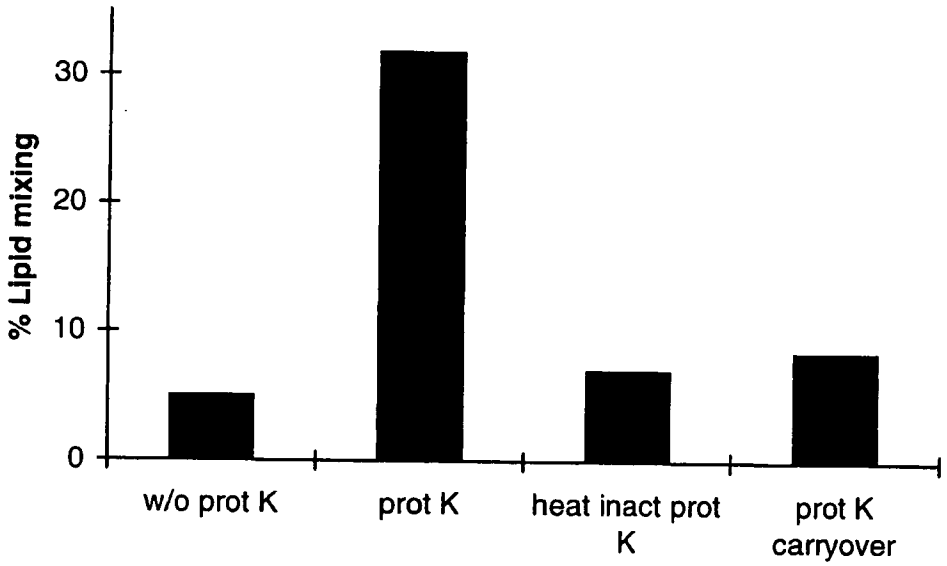

% Lipid mixing
30
20
10
0
w/o prot K
prot K
heat inact prot K
prot K carryover

Fig. 12

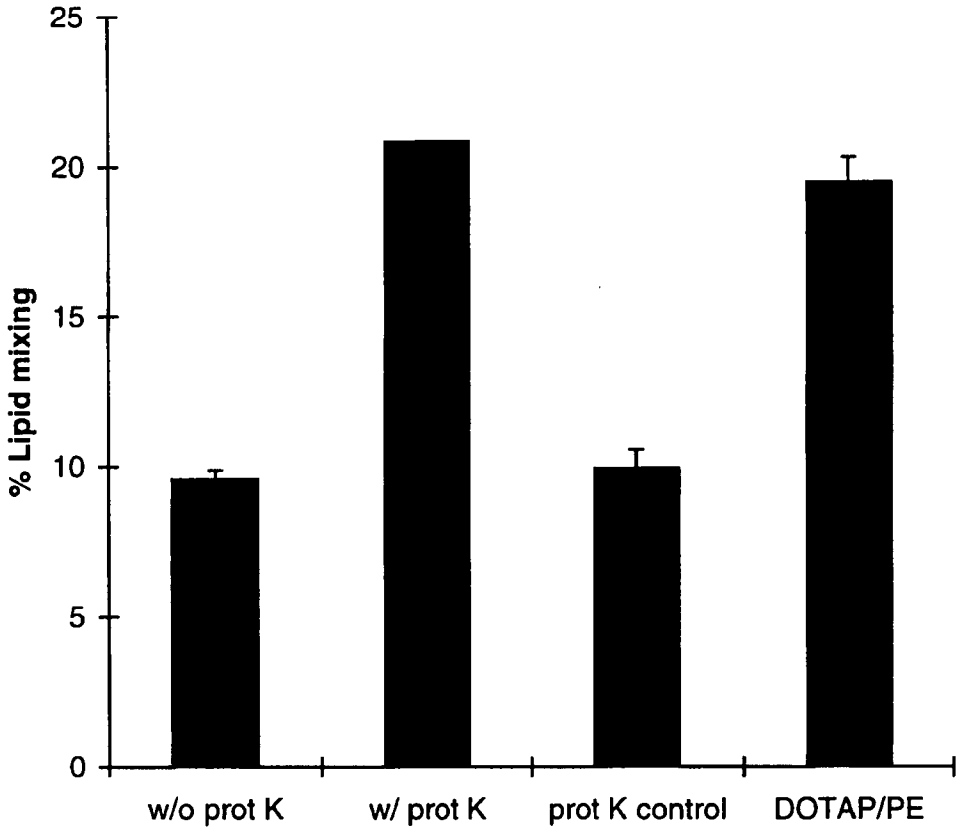

25
20
15
10
5
0
% Lipid mixing
w/o prot K
w/ prot K
prot K control
DOTAP/PE

Fig. 13

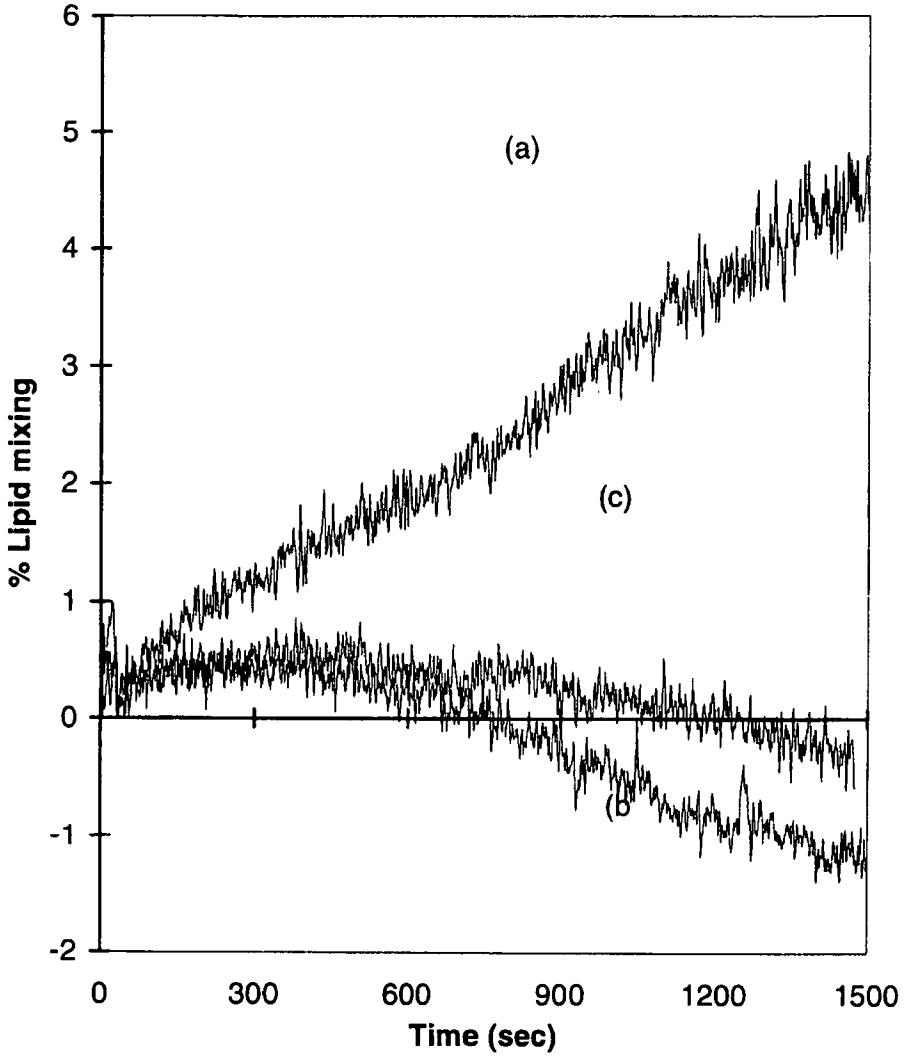


Fig. 14A

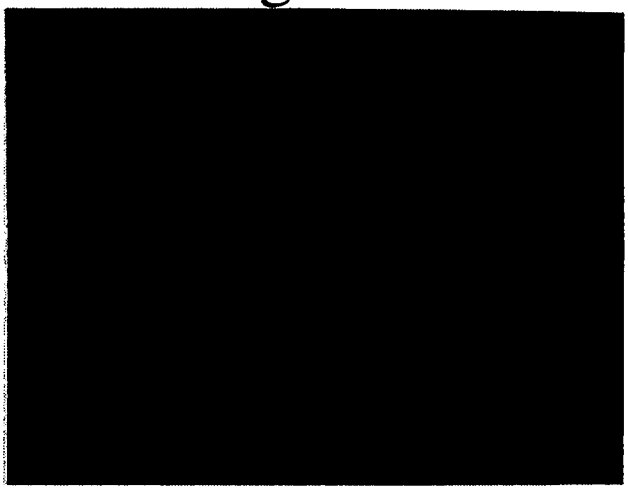


Fig. 14B

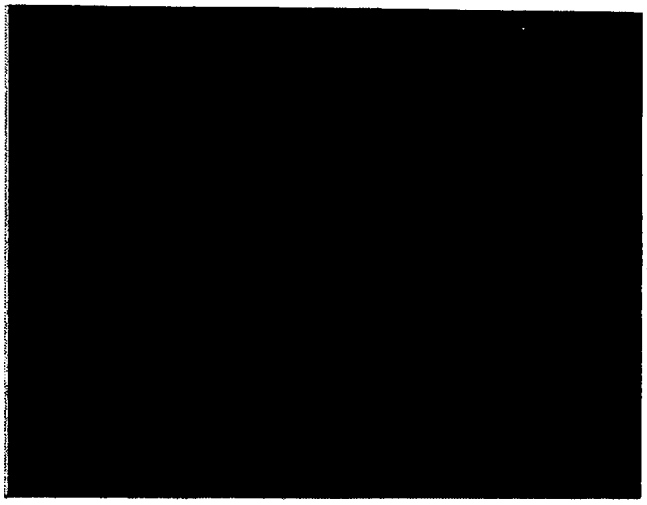


Fig. 14C

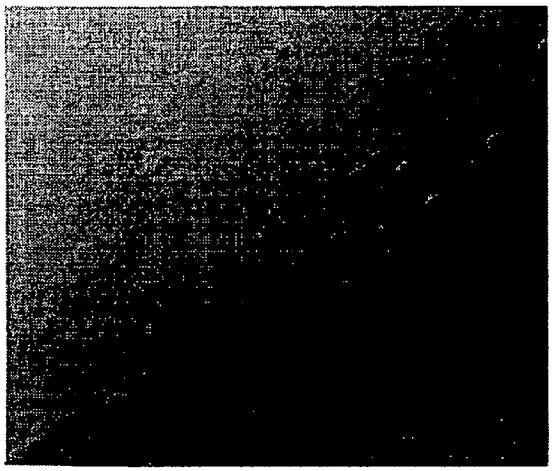


Fig. 14D

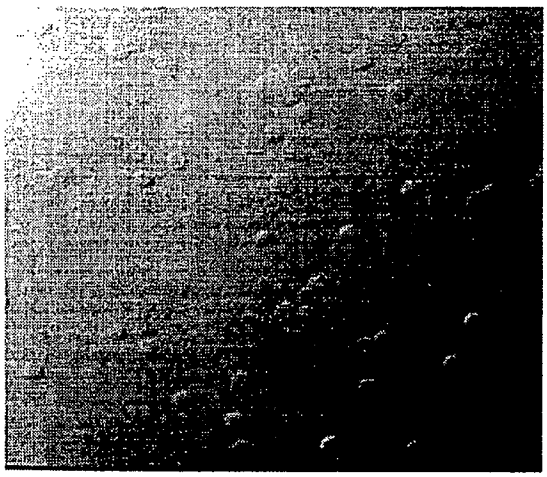


Fig. 15A

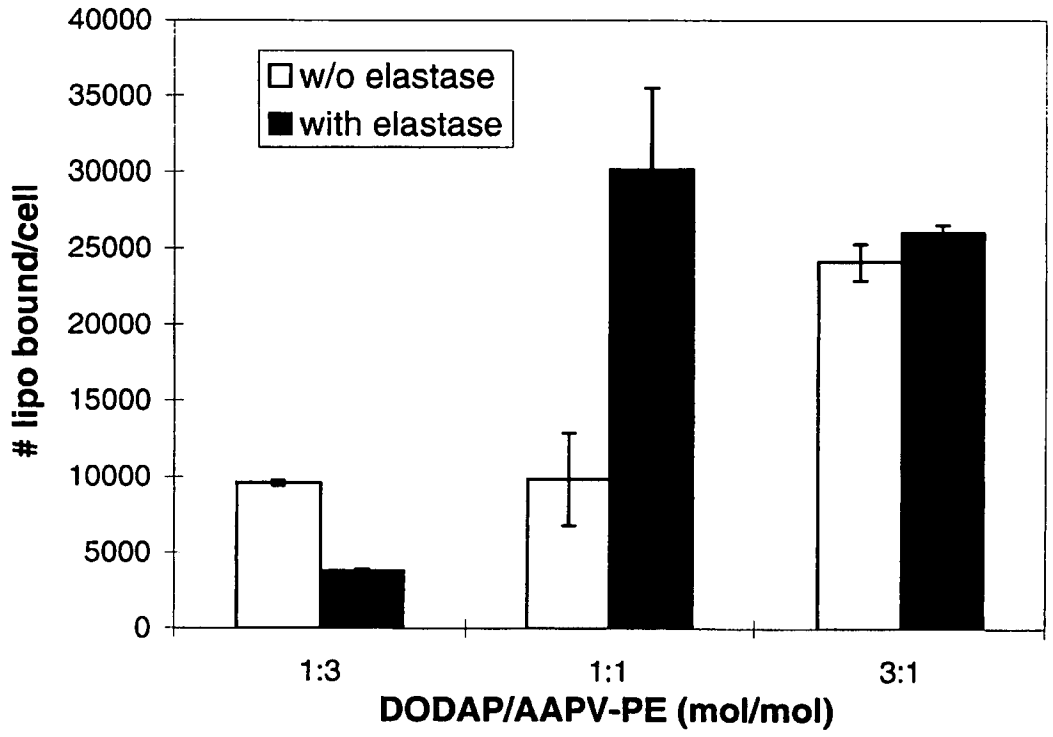

40000
35000
30000
25000
20000
15000
10000
5000
0
lipo bound/cell
w/o elastase
with elastase
1:3
1:1
3:1
DODAP/AAPV-PE (mol/mol)

Fig. 15B

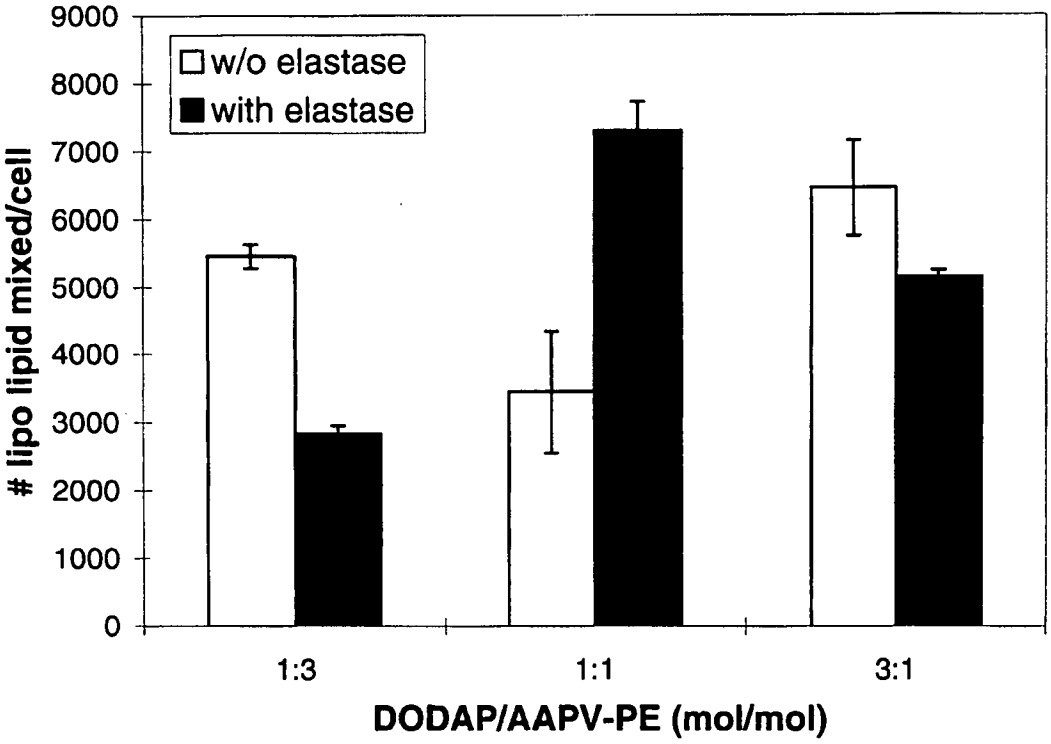

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
lipo lipid mixed/cell
w/o elastase
with elastase
1:3
1:1
3:1
DODAP/AAPV-PE (mol/mol)

Fig. 16A

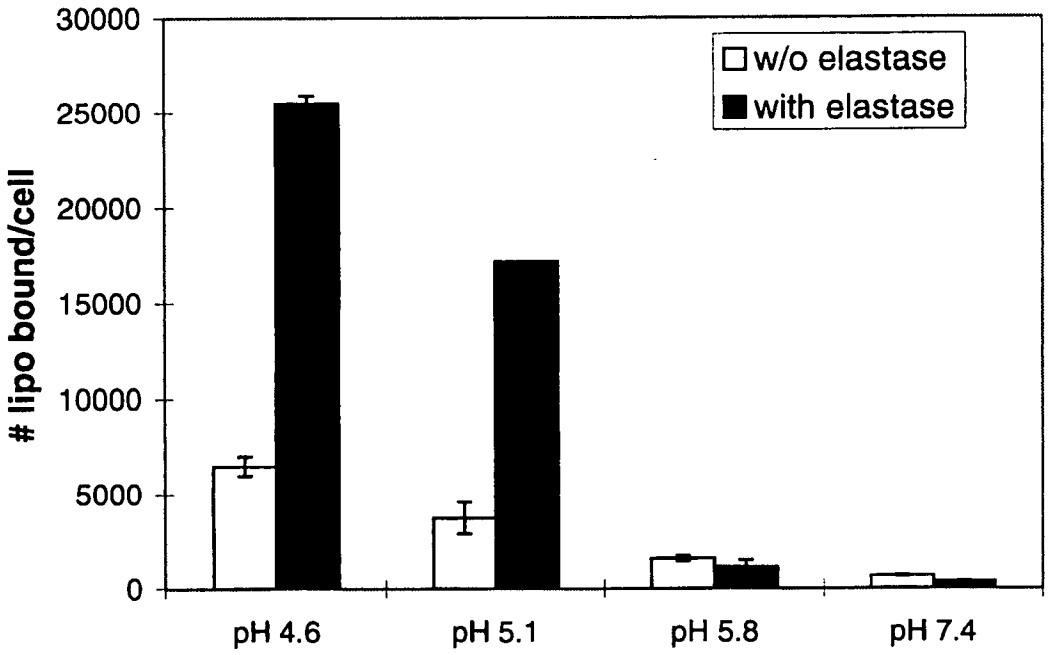
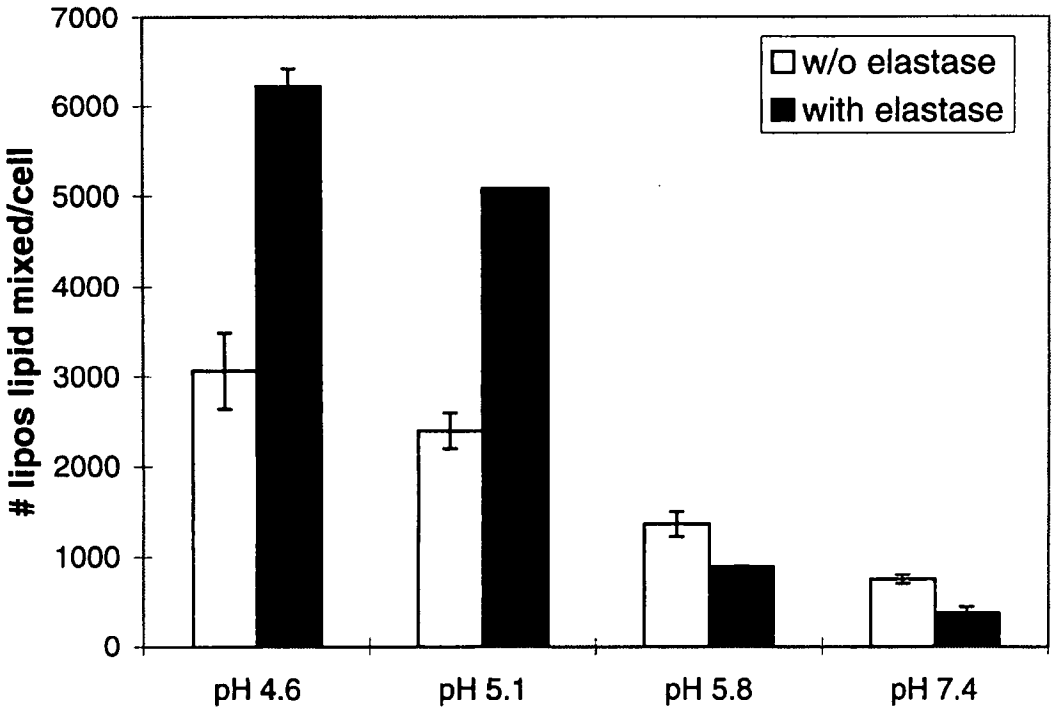

30000
25000
20000
15000
10000
5000
0
lipo bound/cell
w/o elastase
with elastase
pH 4.6
pH 5.1
pH 5.8
pH 7.4

Fig. 16B


7000
6000
5000
4000
3000
2000
1000
0
lipos lipid mixed/cell
w/o elastase
with elastase
pH 4.6
pH 5.1
pH 5.8
pH 7.4

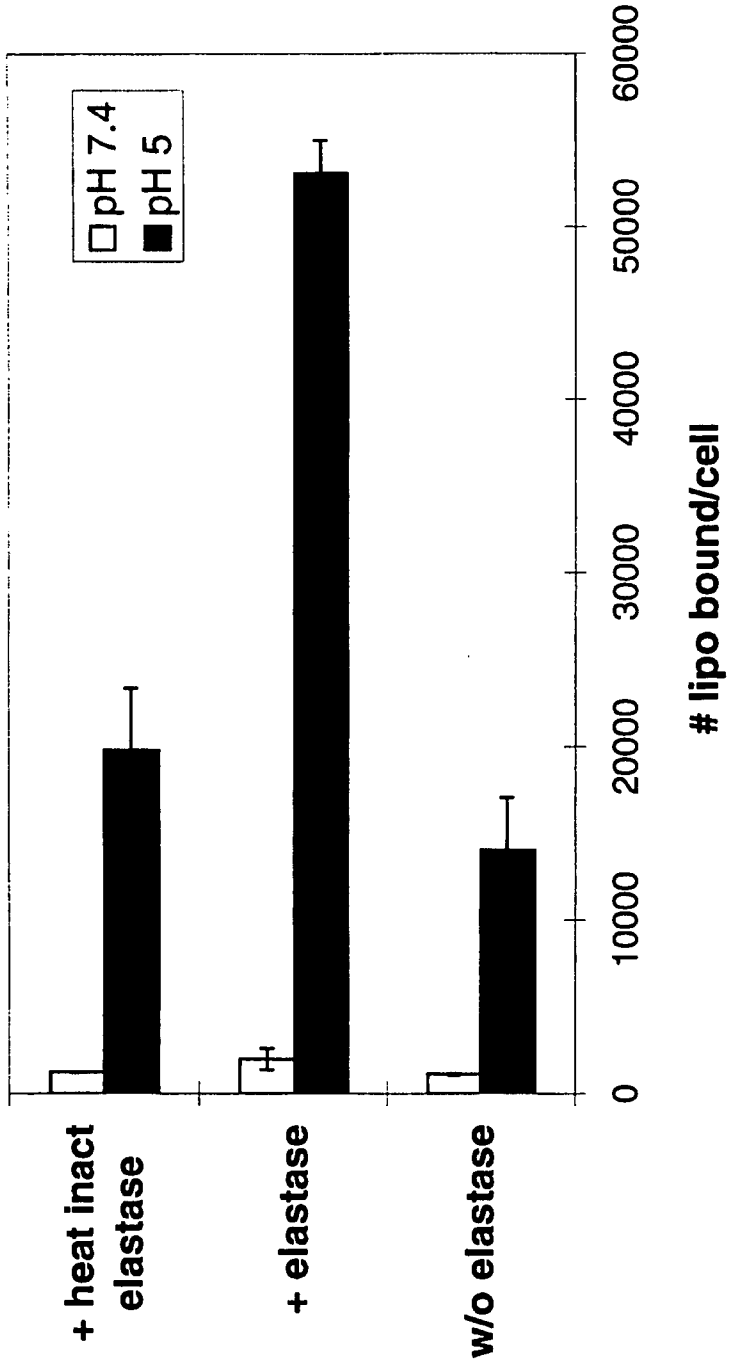

pH 7.4
pH 5
0
10000
20000
30000
40000
50000
60000
lipo bound/cell
+ heat inact elastase
+ elastase
w/o elastase

Fig. 17A

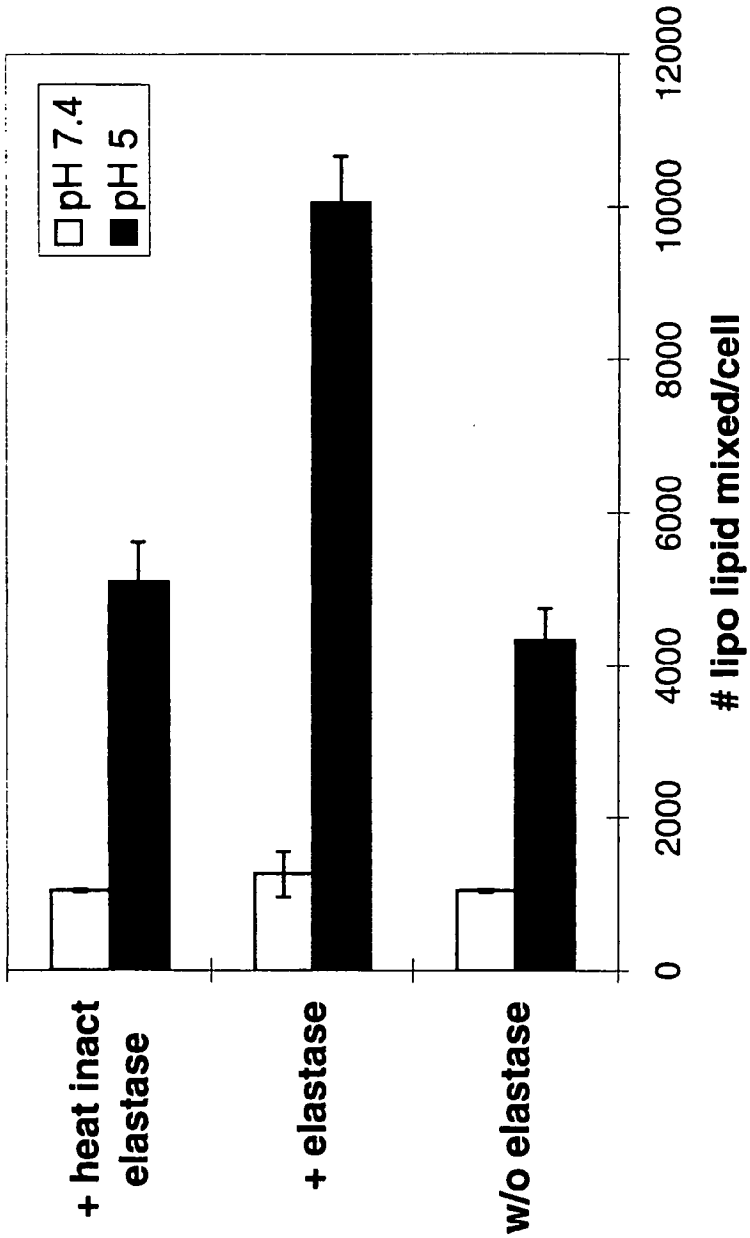
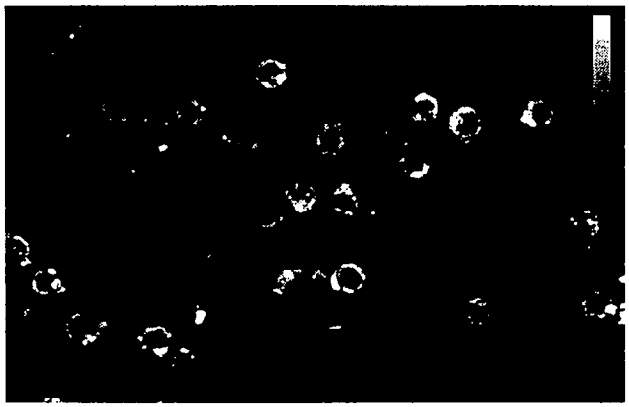

pH 7.4
pH 5
0
2000
4000
6000
8000
10000
12000
lipo lipid mixed/cell
+ heat inact elastase
+ elastase
w/o elastase

Fig. 17B

Fig 18A



with elastase activation

without elastase activation

Fig 18B

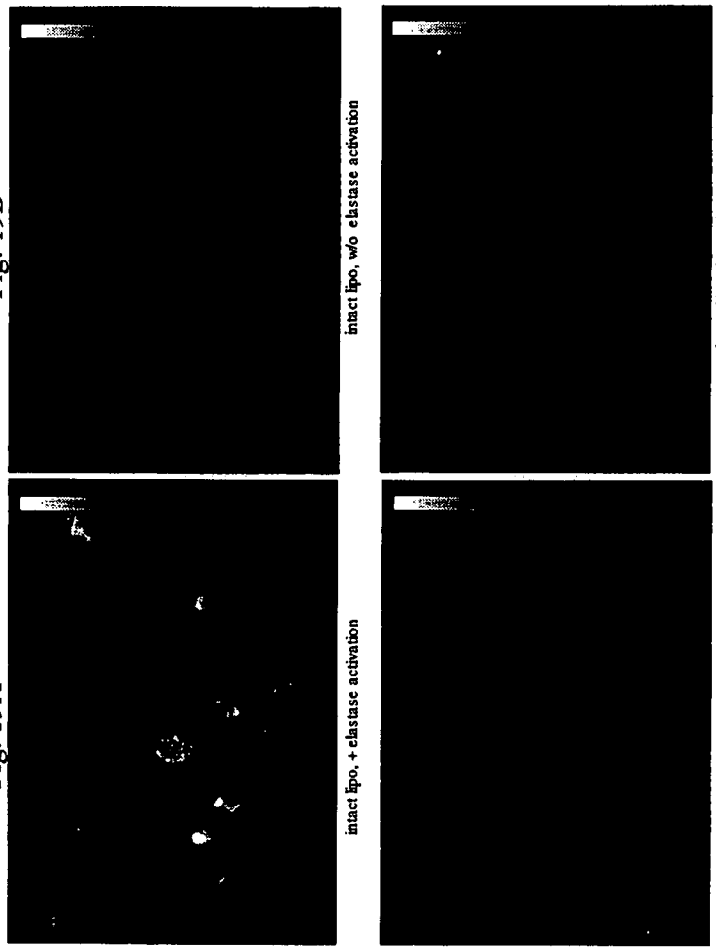


Fig. 19A

Fig. 19B

Fig. 19C

Fig. 19D

Fig. 20

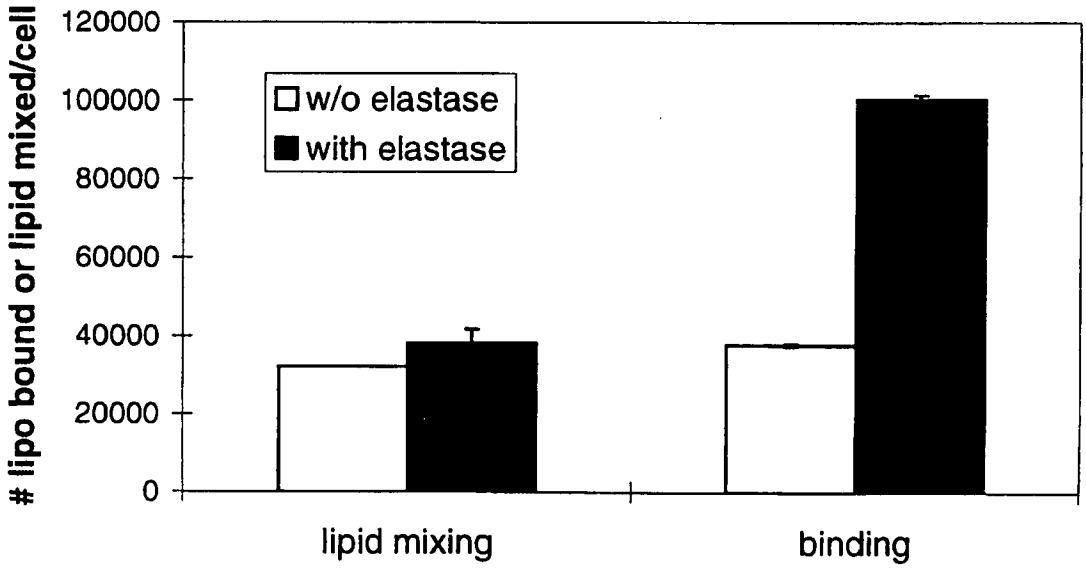

lipo bound or lipid mixed/cell
120000
100000
80000
60000
40000
20000
0
w/o elastase
with elastase
lipid mixing
binding

Fig. 21A

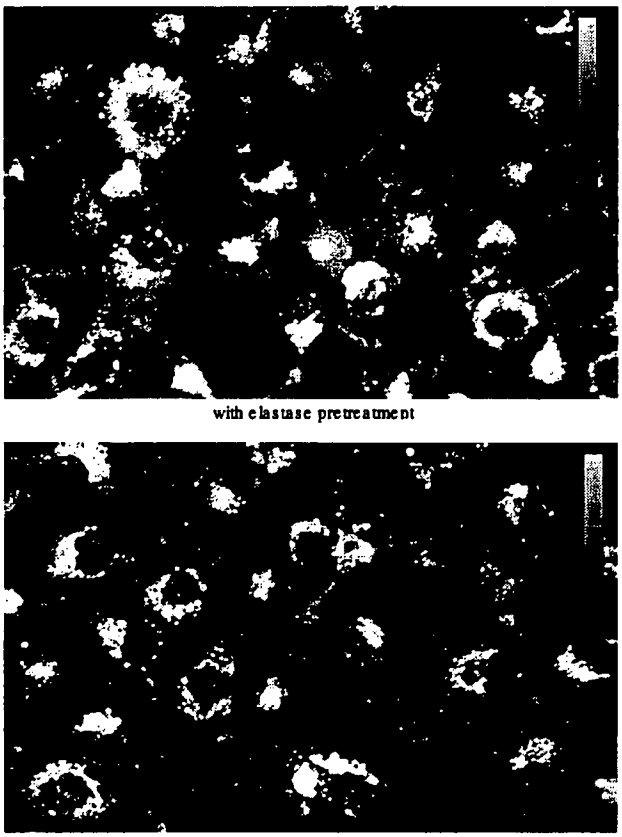
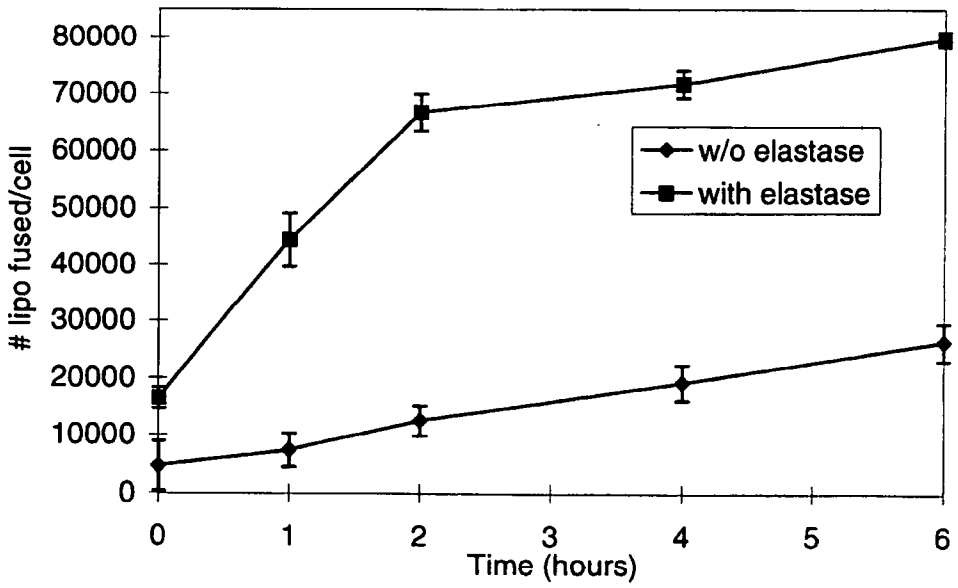


Fig. 21B

Fig. 22


80000
70000
60000
50000
40000
30000
20000
10000
0
lipo fused/cell
0
1
2
3
4
5
6
Time (hours)
w/o elastase
with elastase

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16248

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 48/00; C12N 15/00, 15/63, 15/88; A61K 9/127
US CL :514/44, 435/320.1, 455, 458; 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44, 435/320.1, 455, 458; 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, E	US 6,087,325 A (MEERS et al) 11 July 2000, entire document, especially columns 39 and 40.	1-26
A	US 5,013,556 A (WOODLE et al) 07 May 1991, entire document.	1-26
A	US 4,837,028 A (ALLEN et al) 06 June 1989, entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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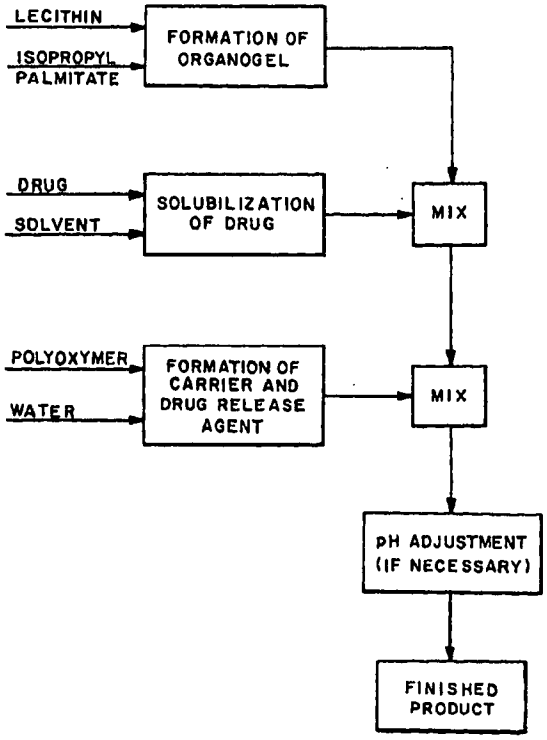
(54) Title: TRANSDERMAL DELIVERY OF MEDICATIONS USING A COMBINATION OF PENETRATION ENHANCERS

(57) Abstract

A composition and procedures for its formation and administration are described, which provide for a convenient, efficacious and simple transdermal administration of medications from a topically applied cream. No transmission through a membrane is involved. The composition incorporates at least two separate penetration enhancers which function synergistically to provide for rapid but controllable transport of the medication from the cream into the skin. The use of a plurality of penetration enhancers, at least one of which facilitates the separation of medication from the cream and at least a second of which alters the structure of the outer layers of skin, particularly the stratum corneum, enhances migration of the drug through the stratum corneum.



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TRANSDERMAL DELIVERY OF MEDICATIONS USING A COMBINATION OF PENETRATION ENHANCERS

BACKGROUND OF THE INVENTION

Field of the Invention:

The invention herein relates to the transdermal delivery of medications to a patient. More particularly it relates to compositions which allow medication molecules to be solubilized and delivered transdermally and to methods for formation of such compositions and for their therapeutic use.

(For convenience herein the terms "drug" and "medication" may be used interchangeably. We wish to emphasize, however, that this invention is applicable to the delivery of any type of compound or molecular species which is intended to be administered to a patient transdermally for a therapeutic or physiological purpose. Whether the material happens to meet a particular specific definition of a "drug" or "medication" or other applicable term is not critical for the purposes of this invention, and the invention should not be limited by the particular term applied to the material being administered.)

Description of the Prior Art:

In the past the delivery of medications transdermally to a patient has been limited to administration by transcutaneous injection or by transdermal migration from a patch placed on the outer surface of the patient's skin. The deficiencies of administration by injection are obvious. With only a few exceptions injections must be administered by trained and qualified medical personnel. The injection itself causes a break in the skin which can lead to infection, despite precautions; an injection needle may itself be contaminated causing infection to the patient; and, course, it is a simple fact that injections are uncomfortable to almost all patients. Further, an injection is normally not "location specific." Rather the injection is made at a location on the body remote from the affected area, and the injected medication must be transported through the body to that location.

This results in losses in transport, so that to administer an effective amount of medication to the affected area, and excess of medication must be injected.

In view of these deficiencies of injection administration, significant effort has been spent in the last few years in seeking alternative methods of transdermal administration of medications. It has been necessary to meet two requirements. First, the method must provide for extended containment of the drug and any carrier while in place on the patient's skin (in effect analogous to containment of the medication and carrier in the reservoir vial of the injection syringe), in a form that does not lend itself either to contamination of the medication and carrier or to loss of the medication and carrier. Second, the systems employed must provide for a regulated and predictable rate of transfer of the medication (with or without the carrier) from the containment device into and through at least some layers of skin to where the medication will be dispersed throughout the affected area of the body.

The only workable prior art embodiment of such a device has been what is commonly known as a "patch." A patch is generally a flat hollow device with a permeable membrane on one side and also some form of adhesive to maintain the patch in place on the patient's skin, with the membrane in contact with the skin so that the medication can permeate out of the patch reservoir and into and through the skin. The outer side the patch is formed of an impermeable layer of material, and the membrane side and the outer side are joined around the perimeter of the patch, forming a reservoir for the medication and carrier between the two layers.

Numerous kinds of medications have been administered through the use of a patch, notably scopolamine for preventing motion sickness, nicotine derivatives intended to discourage an addicted smoker from continuing the smoking habit and estrogen hormones.

Patches have their own set of disadvantages. A principal disadvantage is that, not withstanding the presence of a penetration enhancer, the delivery of

the medication is necessarily limited by the rate of passage of the medication through the patch membrane to the skin. Since the medication is not in contact with the skin while it is enclosed in the patch, whatever length of time is required for the medication to permeate through the skin itself to become effective is necessarily lengthened by the time needed for the medication first to exit from the patch through the membrane. In many cases the membrane permeation rate is the significant rate limiting step of speed of effectiveness of a particular medication, and can render patch administration essentially ineffective because the medication cannot reach the patient's system rapidly enough to be efficacious. In addition, the adhesive which is intended to secure the patch to the patient's skin can fail, so that the patch disengages from the skin before completion of the transfer of the medication, resulting in loss of that quantity of medication which remains within the patch's reservoir.

Various methods have been used to increase skin permeation of medications, including penetration enhancers, pro drugs, superfluous vehicles, iontophoresis, phonophoresis and thermophoresis. For the purposes of this invention, only the penetration enhancers are relevant. Ideal enhancers have no irritancy and toxicity to the skin, and the whole body, together with having high enhancing effects. Enhancers themselves should be phisiochemically stable and not have pharmacologic effects, and preferably should not have smell, color, or taste. A typical example of an enhancer is disclosed in U.S. Patent No. 4,783,450 (to Fawzi et al.) in which lecithin is used for penetration enhancement.

The stratum corneum provides the principal barrier to the percutaneous penetration of topically applied substances. It is the most superficial cutaneous layer and is a horny layer that consists of flat, scalelike "squames" made up of the fibrous protein keratin. The squames are continually being replaced from below by epidermal cells that die in the process of manufacturing keratin. It is unlikely that the emulsified fat on the skin surface greatly affects permeability.

However, vehicles can control, to a great extent, the rate of penetration of drugs that are applied to the skin. The intercellular lipids may be important for the permeability barrier in skin.

It is known that some combinations of enhancers and vehicles act synergistically, such as the combination of ethanol as a vehicle for the enhancer laurocapram. However, many combinations are not synergistic; for instance, n-decylmethylsulfoxide lowers the zeta potential of the skin, and thus enhancement due to conduction flow (iontophoresis) is minimized. in the past, synergism of combinations could not be predicted.

Further, one must differentiate between penetration enhancer which act to improve the ability of the medication to pass through a patch membrane to reach the skin, and those which act to enhance the separation of the medication from its carrier matrix or to enhance the diffusion of the medication into and through the skin.

However, notwithstanding the various deficiencies mentioned, administration by injection or by patch remain only by viable transdermal administration techniques known to the prior art.

SUMMARY OF THE INVENTION

We have now developed a system that provides for a convenient, efficacious and simple system for transdermal administration of medications in which the medication is present in a composition for direct application to the skin, commonly in the form of a cream or similar material. The transdermal administration of the drug is therefore not hindered by having to penetrate a patch membrane, since the cream and its medication content are directly in contact with the skin and the medication needs only to separate from the cream in order to be available for transdermal migration. In addition, since the composition is in the form of a cream or other viscous moldable and spreadable material, the drug may be effectively administered by application of the cream

to many bodily areas where a patch either will not fit or cannot be shaped to conform to the skin contours.

(As with the use of the terms "medication" and "drug," our invention is not to be limited by the term used to describe the physical properties of the composition herein. We will for convenience use the term "cream," but other terms such as "gel," "lotion," "paste" and the like also could be applicable. As will be seen from the description below, the physical nature of the composition containing the medication and to be applied to the patient's skin will be defined by functional parameters, rather than being limited by an arbitrary descriptive term.)

A key element in the success of the present invention is our discovery that the use of at least two separate penetration enhancers of defined function results in a synergism which provides rapid but controllable separation of the medication from the cream and its penetration into and within or through the skin. At least one of the penetration enhancers acts to facilitate the separation of drug from the carrier within the cream and at least a second penetration enhancer alters the structure of the outer layers of skin, particularly the stratum corneum, such that migration of the drug through the stratum corneum is enhanced and expedited. The medication is thus taken up by the patient's system and is efficacious much more rapidly than would be the case for administration of the medication by means of the prior art patch system. Further, although permeation of the skin does not provide for as rapid administration by the medication as would result from direct injection, the use of the present invention avoids the problems associated with injection administration.

Therefore, in one principal embodiment, the invention is of a composition for diffusional transdermal delivery of medication to a patient, which comprises a medication capable of being administered transdermally; a carrier for the medication; a first penetration enhancer which improves diffusion of the medication into and within the patient's skin; and a second penetration enhancer

which improves diffusion of the medication out of the composition for transdermal migration; the composition having a viscosity in a range such that it may be applied topically and conform to and adhere to the patient's skin for a period of time sufficient for a significant portion of the medication to be delivered transdermally to the patient.

In another principal embodiment, the invention is of a method for the preparation of a therapeutic composition to be transdermally administered which comprises solubilizing a medication capable of being administered transdermally; forming an organogel comprising a first penetration enhancer which improves diffusion of the medication into and within the patient's skin, and a carrier for the solubilized medication; forming a polymeric component comprising a second penetration enhancer which improves diffusion of the medication out of the composition for transdermal migration; and blending the solubilized medication, organogel and polymeric component to form the composition having a viscosity in a range such that it may be applied topically and conform to and adhere to the patient's skin for a period of time sufficient for a significant portion of the medication to be delivered transdermally to the patient.

In yet another principal embodiment, the invention is of a method for the transdermal administration of a medication which comprises solubilizing a medication capable of being administered transdermally; forming an organogel comprising a first penetration enhancer which improves diffusion of the medication into and within the patient's skin, and a carrier for the solubilized medication; forming a polymeric component comprising a second penetration enhancer which improves diffusion of the medication out of the composition for transdermal migration; blending the solubilized medication, organogel and polymeric component to form the composition having a viscosity in a range such that it may be applied topically and conform to and adhere to the patient's skin for a period of time sufficient for a significant portion of the medication to be delivered transdermally to the patient; and applying the composition to the skin

of a patient for the period of time and allowing the medication to diffuse out of the composition and through the skin, such that the medication is taken up by the body of the patient and acts therapeutically on the patient.

In preferred embodiments the first penetration enhancer is a lecithin organogel formed with isopropyl palmitate or isopropyl myristate, and the second penetration enhancer is a polyoxymer, preferably a polyoxyalkylene derivative of propylene glycol. A wide variety of medications can be delivered by this invention. Further, while the invention herein is described in terms of the minimum number of synergistically acting penetration enhancers (i.e., two), it will be understood that additional penetration enhancers can also be present. Thus there may be more than one enhancer which operates with a specific mechanism, or there may be additional enhancers which provide yet other modes of operation, or both.

The methods and compositions described herein provide a unique and highly effective technique for administering medication directly to an affected area of the body with the minimum amount of medication and with the avoidance of unwanted side effects. Unlike administration by injection or orally, the transdermal administration herein is site specific; the cream is applied to the skin directly at the affected area of the body. There are therefore no losses of medication during transport from a remote application site. Similarly, the long delays in having an effective quantity of the medication reach the affected area of the body, which are inherent in injection and oral administration, are entirely eliminated in the present invention.

The present method also avoids unwanted side effects. For instance, in oral administration of a medication, the medication itself can adversely affect the gastrointestinal tract as it is swallowed and dissolved for assimilation into the circulatory system. Those skilled in the art are well familiar with the common caution required for many oral medications that they must be administered only in conjunction with a meal, or, conversely, that they cannot be administered in

the presence of specific types of food products, such as dairy products. These cautions are necessary since the orally administered medication's efficacy will be adversely affected by certain foods, or the person's gastrointestinal tract will be irritated by the medication if the latter is not diluted by the presence of food in the gastrointestinal tract. Such considerations are, of course, entirely absent in the present invention, where the same medications can be easily and conveniently administered transdermally without incurring such side effects.

Further, the transdermal administration avoids the "first pass effect," which often results when a medication is administered orally and thus has to pass through various organs, including the liver, before reaching the affected area of the body. These organs can absorb or chemically alter significant quantities of the passing medication, thus requiring that large excess quantities of the medication by administered initially to insure that an effective quantity of the medication will ultimately reach the affected area of the body. Since in this method the medication commonly passes through the skin directly to the affected site, there is no problem of loss in intermediate organs, and therefore excessive quantities of medication do not need to be delivered to counter such losses. (As an example, ketoprofen is commonly administered orally in quantities of about 50-75 mg per dose for the desired efficacy. In the present invention, however, an equally effective dose of ketoprofen can be delivered by topical transdermal administration of only 3 mg.)

Finally, since the present invention is site specific, the depth of delivery of the medication can be readily controlled, as contrasted to injection delivery.

BRIEF SUMMARY OF THE DRAWING

The single Figure of the drawing is a flow chart illustrating schematically formulation of a preferred embodiment of a composition of this invention.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS

The unique compositions of the present invention require a specific sequence of steps in their formation if a therapeutically effective and pharmaceutically compatible composition is to be obtained. This is best understood by reference to the Figure of the drawing.

The basic composition of this invention is a mixture of an organogel, a solubilized medication or drug and a carrier combined with a drug release agent. Penetration enhancement is provided by the organogel and by the release agent.

In the exemplary process as illustrated in the Figure, an organogel is formed, in this example from lecithin and isopropyl palmitate. These two materials are thoroughly blended and mixed until a substantially uniform gel structure forms. The organogel, which is the base for the cream composition, should be formed at the time that the composition is to be formulated. The drug or medication is solubilized with a solvent, such as water, alcohol or other appropriate solvent, again by mixing in a known manner. When it is desired to start formation of the actual composition, the solubilized drug is mixed thoroughly into the organogel matrix, again by conventional mixing techniques. The technique used will of course be such that the organogel's structure is not broken down. Finally, a carrier, such as water or alcohol, and a drug release agent, such as a polyoxymer, are blended. The carrier/release agent mixture can be made up in large lots and stored under refrigerator until needed, at which time an appropriate quantity can be taken for and the remainder retained in refrigerated storage. The carrier/release agent mixture is then mixed with the drug/organogel mixture to produce the final "cream" composition. Details will be provided below.

Considering first the organogel, the blend of the two components will be in the range of from about 25% to 75% of the lecithin component, the remainder being the fatty acid ester component. (Unless stated otherwise, all percentages,

parts and concentrations are by weight.) The "lecithin component" may be lecithin, any comparable fatty acid phospholipid emulsifying agent, such as fatty acids and their esters, cholesterol, tri-glycerides, gelatin, acacia, soybean oil, rapeseed oil, cottonseed oil, waxes or egg yolk, or any other material which acts in the same manner as lecithin.

The other component is an organic solvent/emollient, particularly including fatty acid esters, of which the esters of the saturated alkyl acids are preferred. The preferred solvent/emollient in the present invention is isopropyl palmitate or isopropyl myristate. However, there are numerous compounds available which exist in liquid form at ambient temperatures and will function in a manner equivalent to the fatty acid esters. These are all quite well known and described. They include, but are not limited to, the following:

Ethanol
Propylene glycol
Water
Sodium oleate
Leucinic acid
Oleic acid
Capric acid
Sodium caprate
Lauric acid
Sodium laurate
Neodecanoic acid
Dodecylamine
Cetyl lactate
Myristyl lactate
Lauryl lactate
Methyl laurate
Phenyl ethanol
Hexamthhylene lauramide
Urea and derivatives
Dodecyl n,n-dimethylamino acetate
Hydroxyethyl lactamide
Phyophatidylcholine
Sefsol-318 (a medium chain glyceride)
Isopropyl myristate
Isopropyl palmitate
Surfactants (including):
    polyoxyethylene (10) lauryl ether
    diethyleneglycol lauryl ether
Laurocapram (azone)
    (1;1-dodecylazacycloheptan-2-one)
Acetonitrile
1-decanol
2-pyrrolidone
N-methylpyrrolidone
N-ethyl-1-pyrrolidone
1-methyl-2-pyrrolidone
1-lauryl-2-pyrrolidone
Sucrose monooleate
Dimethylsulfoxide
Decylmethylsulfoxide
Acetone
Polyethylene glycol (100-400mw)
Dimethylacetamide
Dimethylformamide
Dimethylisosorbide
Sodium bicarbonate
Various C_7 to C_{16} alkanes
Mentane
Menthone
Menthol
Terpinene
D-terpinene
Dipentene
N-nonalol
Limonene
Ethoxy diglycol

This combination of the phospholipid emulsifying agent and the fatty acid or fatty acid ester or equivalent thereof forms an organogel. In the example referred to in the Figure, the organogel will be a lecithin organogel, which is both isotropic and thermally reversible. At temperatures greater than about 40°C the organogel will become a liquid and its viscosity will be greatly reduced. Water can be also be added to control the viscosity of the organogel. The organogel serves as one of the penetration enhancers in the cream, and acts on the stratum corneum of the skin to promote interaction between the phospholipids of the cream and the phospholipids of the skin. This causes a disruption in the normal regular arrangement of layers in lipids in the stratum corneum so that openings are created which then allow the drug to pass more easily through the skin. The organogel will be compatible with a wide variety of lipophilic, hydrophilic and amphoteric drugs and medications.

Using the above-described lecithin organogel and its components as an example, the properties needed for inclusion of a components in this invention will be evident. The various compounds, polymers, etc. comprising the organogel, the solubilized drug and the carrier/polyoxymer components must all be compatible with each other, so that chemical reactions do not occur which would adversely affect the efficacy or safety of the cream composition; they must be mutually soluble so that they can be mixed and blended to a uniform consistency; they must be such that the resulting cream composition has a viscosity under ambient conditions which is low enough to allow it to be applied easily and smoothly to the skin, but not so low that the cream acts as at least in part like a liquid and cannot be retained on the skin where it is applied; they must not be toxic, irritating or otherwise harmful to the patient; they must be sufficiently stable that the overall composition will have a reasonable shelf life and service life; and, as a practical matter, they must be available at reasonable cost. Thus, it will generally be found that the characteristics of a drug or medication which make it difficult to administer transdermally through the present

system include its having low stability, particularly at ambient temperatures; not being soluble in the composition; having high molecular weight resulting in difficulty penetrating the stratum corneum, even with the enhanced openings; and/or causing an adverse reaction with the one or more skin layers.

The drug or medication which is to be administered usually must be solubilized in a solvent to enable it be blended properly with the organogel and the carrier/release agent. Typical solvents for such use include water, the low molecular weight alcohols and other low molecular weight organic solvents. Solvents such as water, methanol, ethanol and the like are preferred. The purpose of solubilizing is to enable the medication to become properly dispersed in the final cream. It is possible that a few drugs or medications might themselves be sufficiently soluble in the cream that a solvent, and therefore a separate solubilizing step, would not be needed. For the purpose of this description, therefore, the term "solubilized" drug or medication shall be considered to include those drugs or medications which can be dispersed or dissolved into the cream with or without the presence of a separate solvent. Usually the amount each of medication and solvent which will be present, based on the entire composition, will be in the range of up to <1%-20%, with the preferred concentration of each being about 10%. The concentrations of both need not be identical.

A wide variety of drugs may be transported by this method and through this type of composition. Typical of the various drugs which can be successfully incorporated into the present composition and transdermally transported include the following classes of substances:

- Antidiabetic Agents
  - Sulfonylureas
    - Acetohexamide
    - Chlorpropamide
    - Tolazamide
    - Tolbutamide
    - Glipizide
    - Glyburide
    - Glimepiride
  - Metformin
  - Acarbose
  - Insulin
- Glucose Elevating Agents
  - Diazoxide
  - Glucose
- Thyroid Hormones
  - Levothyroxine
  - Liothyronine
  - Thyroid USP
  - Thyroglobulin
  - Liotrix
- Thyroid Drugs
  - Iodine
  - Propylthiouracil
  - Methimazole
- Parathyroid Drugs
  - Calcitonin
  - Etidronate
  - Pamidronate
  - Alendronate
  - Gallium Nitrate
- Vitamins
  - Vitamin A
  - Vitamin D
  - Vitamin E
  - Vitamin B1
  - Vitamin B2
  - Vitamin B3
  - Vitamin B6
  - Vitamin B12
  - Vitamin C (con't. next column)
  - Multivitamin Preparations
  - Vitamin Combinations
- Antihyperlipidemic Agents
  - Fluvastatin
  - Lovastatin
  - Pravastatin
  - Simvastatin
  - Probucol
  - Niacin
  - Dexothyroxine
  - Clofibrate
  - Gemfibrozil
- Cardiac Drugs
  - Cardiac Glycosides
    - Digitoxin
    - Digoxin
  - Antianginal Agents
    - Nitroglycerin
    - Isosorbide Dinitrate
    - Isosorbide Mononitrate
  - Antiarrhythmic Agents
    - Moricizine
    - Quinidine
    - Procainamide
    - Disopyramide
    - Lidocaine
    - Tocainide
    - Mexiletine
    - Flecanide
    - Encainide
    - Amiodarone
- Respiratory Drugs
  - Bronchodilators
    - Albuterol
    - Metaproterenol
    - Terbutaline
    - isoproterenol
    - Ephedrine
    - Theophylline
    - Dyphylline

Nasal Decongestants
- Phenylpropanolamine
- Pseudoephedrine
- Phenylephrine
- Ephedrine
- Naphazoline
- Oxymetazoline
- Tetrahydrozoline
- Xylometazoline
- Propylhexedrine

Gastrointestinals
- Sucralafate
- Metoclopramide
- Cisapride
- Laxatives
- Mesalamine
- Olsalazine
- Antidiarrheals
- Famotidine
- Nizatidine
- Cimetadine
- Rantadine
- Omeprazol
- Cifapride

Miscellaneous
- Finasteride
- Lamsoprazole
- Papaverine
- Prostaglandins

Amphetamines
- Dextroamphetamine

Anorexiants
- Phentermine
- Benzphetamine
- Phendimetrazine
- Diethylpropion
- Mazindol
- Fenfluramine
- Dexfenfluramine

Antirheumatic Agents
- Gold Compounds
- Penicillamine
- Azathioprine
- Methotrexate

Agents for Gout
- Probenecid
- Sulfinpyrazone
- Allopurinol
- Colchicine

Agents for Migraine
- Sumatriptan
- Methysergide
- Ergotamine Derivatives

Sedatives and Hypnotics
- Zolpidem
- Paraldehyde
- Chloral Hydrate
- Acetylcarbromal
- Glutethimide
- Ethchlorvynol
- Ethimate
- Temazepam
- Estazolam
- Flurazepam
- Quazepam
- Triazolam
- Phenobarbital
- Mephobarbital
- Amobarbital
- Butabarbital
- Secobarbital
- Pentobarbital

Antianxiety Agents
- Meprobamate
- Alprazolam
- Chlordiazepoxide
- Clonazepam
- Clorazepate
- Diazepam
- Halazepam
- Lorazepam
- Oxazepam (con't. next page)

- Prazepam
- Buspirone
- Hydroxyzine
- Doxepin
- Chlormezanone

Anticonvulsants
- Phenytoin
- Mephenytoin
- Ethotoin
- Ethosuximide
- Methsuximide
- Phensuximide
- Paramethadione
- Trimethadione
- Clonazepam
- Clorazepate
- Valproic Acid
- Lamotrigine
- Primidone
- Gabapentin
- Phenacemide
- Carbamazepine
- Phenobarbitol

Antidepressants
- Amitryptyline
- Clornipramine
- Doxepin
- Imipramine
- Trimipramine
- Amoxapine
- Desipramine
- Nortriptyline
- Protriptyline
- Venlafaxine
- Maprotiline
- Trazodone
- Bupropion
- Fluoxetine
- Paroxetine
- Sertraline
- Fluvoxamine
- Tranylcypromine
- Phenelzine
- Nefazodone

Antipsychotic Agents
- Chlorpromazine
- Promazine
- Triflupromazine
- Thioridazine
- Mesoridazine
- Acetophenazine
- Perphenazine
- Fluphenazine
- Trifluoperazine
- Chlorprothixene
- Thiothixene
- Haloperidol
- Molindone
- Loxapine
- Clozapine
- Riperidone
- Pimozide
- Prochlorperazine

Other Psychotherapeutic Agents
- Lithium
- Methylphenidate
- Tacrine
- Pemoline

Antimicrobials
- Antibacterials
  - Penicillins
  - Cephalosporins
  - Carbapenems
  - Monobactams
  - Chloramphenicoi
  - Fluoroquinolones
  - Tetracyclines
  - Macrolides
  - Spectinomycin
  - Vancomycin
  - Lincosamides
  - Aminoglycosides
  - Colistin
  - Polymixin B
  - Bacitracin
  - Novobiocin
  - Metronidazoie

- Antifungals
  - Flucytosine
  - Nystatin
  - Miconazole
  - Ketoconazole
  - Amphotericin B
  - Griseofulvin
  - Fluconazole
  - Itraconazole
- Sulfonamides
  - Sulfadiazine
  - Sulfacytine
  - Sulfamethoxazole
  - Suflamethiazole
- Antimalarials
  - Quinine Sulfate
  - Mefloquine
  - Quinacrine
  - Doxycycline
  - 4-Aminoquinolone Compounds
  - 8-Aminoquinolone Compounds
  - Folic Acid Antagonists
- Antituberculous Drugs
  - Isoniazid
  - Rifampin
  - Rifabutin
  - Ethambutol HCl
  - Pyrazinamide
  - Aminosalicylate Sodium
  - Ethionamide
  - Cycloserine
  - Streptomycin Sulfate
  - Capreomycin
- Amebicides
  - Paromomycin
  - Iodoquinol
  - Metronidazole
  - Emetine
  - Chloroquine
- Antivirals
  - Famciclovir
  - Stavudine
  - Zidovudine
  - Ribavarin (con't. next column)
  - Amantadine
  - Foscarnet
  - Didanosine
  - Acyclovir
  - Ganciclovir
  - Zalcitabine
  - Rimantadine
- Miscellaneous Anti-infectives
  - Trimethoprim
  - Trimethoprim-Sulfamethoxazole
  - Erythromycin-Sulfisoxazole
  - Furazolidone
  - Pentamidine
  - Eflornithine
  - Atovaquone
  - Trimetrexate Glucuronate
- Leprostatics
  - Dapsone
  - Clofazime
- Antihelmintics
  - Mebendazole
  - Diethylcarbamazine Citrate
  - Pyrantel
  - Thiabendazole
  - Piperazine
  - Quinacrine
  - Niclosamide
  - Oxamniquine
  - Praziquantel

- Antihistamines
  - Diphenhydramine
  - Chlorpheniramine
  - Pyrilamine
  - Doxepin
  - Carbinoxamine
  - Clemastine
  - Tripelennamine
  - Brompheniramine
  - Dexchlorpheniranune
  - Triprolidine
  - Methdilazine
  - Promethazine
  - Trimeprazine (con't. next page)

Hydroxyzine HCl
Azatadine
Cyproheptadine
Phenindamine
Astemizole
Loratadine
Terfenadine
Cetirizine

Antimetabolites
5-Fluorouracil
6-Mercaptopurine
Mycophenolic Acid
Methotrexate
Cytarabine
Floxuridine
Thioguanine

Anticholinergics
Atropine
Scopolamine
Homatropine
Tropicamide
Pirenzepine
Isopropamide
Propantheline
Methscopolamine
Methantheline
Trihexyphenidyl
Benztropine
Biperiden

Steroidal Antiinflammatory Agents
Cortisone
Hydrocortisone
Hydrocortisone Acetate
Prednisone
Prednisolone
Triamcinolone
Methylprednisolone
Dexamethasone
Betamethasone
Clobetasol
Diflorasone
Halobetasol
Amicinonide (con't. next column)
Desoximetasone
Fluocinolone
Halcinonide
Clocortolone
Flurandrenolide
Fluticasone
Mometasone
Aclometasone
Desonide
Fludrocortisone

Local Anesthetics
Dibucaine
Lidocaine
Benzocaine
Butamben Picrate
Tetracaine
Dyclonine
Pramoxine
Prilocaine

Antiplatelet Drugs
Dipyridamole
Ticlopidine
Warfarin
Coumarin

Non-steroidal Antiinflammatory Agents
Fenoprofen
Ibuprofen
Flurbiprofen
Ketoprofen
Naproxen
Oxaprozin
Diclofenac
Etodalac
Indomethacin
Ketorolac
Nabumetone
Sulindac
Tolmentin
Meclofenamate
Flufenamic Acid
Mefenamic Acid
Meclofenamic Acid
Piroxicam
Salicylates (con't. next page)

- Diflunisal
- Indomethacin
- Phenylbutazone
- Oxyphenbutazone
- Sulfinpyrazone
- Allopurinol
- Penicillamine
- Colchicine
- Probenicid

Sunscreen Agents

- Oxybenzone
- Dioxybenzone
- p-Aminobenzoic Acid
- Ethyl Dihydroxy Propyl PABA
- Padimate 0
- Glyceryl PABA
- Cinoxate
- Ethylhexyl p-methoxycinnamate
- Octocrylene
- Octyl Methoxycinnamate
- Ethylhexyl salicylate
- Homosalate
- Octyl Salicylate
- Menthyl Anthranilate
- Digalloyl Trioleate
- Avobenzone

Muscle Relaxants

- Carisoprodol
- Chlorphenesin
- Chlorzoxazone
- Cyclobenzaprine
- Metaxalone
- Methocarbamol
- Orphenadrine
- Diazepam
- Baclofen

Antihypertensives

- Beta-Blockers
  - Propranolol
  - Acebutolol
  - Betaxolol
  - Bisoprolol
  - Esmolol (con't. next column)
  - Carteolol
  - Nadolol
  - Penbutolol
  - Pindolol
  - Sotalol
  - Timolol
  - Labetalol
- Ace Inhibitors
  - Benazepril
  - Captopril
  - Enalapril
  - Fosinopril
  - Lisinopril
  - Moexipril
  - Quinapril
  - Ramipril
- Calcium Channel Blockers
  - Diltiazem
  - Verapamil
  - Nifedipine
  - Felodipine
  - Nicardipine
  - Nimodipine
  - Nisoldipine
  - Isradipine
  - Bepridil
  - Amlodipine
  - Nisoldipine
- Alpha Blockers
  - Methyldopa
  - Clonidine
  - Phentolamine
  - Guanabenz
  - Phenoxybenzamine
  - Guanfacine
  - Yohimbine
  - Reserpine
  - Guanethidine
  - Guandrel
  - Doxazosin
  - Prazosin
  - Terazosin
- Vasodilators
  - Hydralazine
  - Minoxidil
  - Nitroglycerin (con't. next page)

Isosorbide Dinitrate
Isosorbide Mononitrate
Papaverine

Diuretics
Thiazides
Loop Diuretics
Spironolactone
Triamterene
Acetazolamide
Methazolamide
Dichlorphenamide

Antiemetics
Chlorpromazine
Triflupromazine
Perphenazine
Prochlorperazine
Promethazine
Thiethylperazine
Metoclopramide
Cyclizine
Meclizine
Buclizine
Dimenhydrinate
Trimethobenzamide
Scopolamine
Diphenidol
Benzquinamide
Hydroxyzine

Analgesics
Codeine
Hydrocodone
Hydromorphone
Morphine (con't. next column)
Oxymorphone
Oxycodone
Meperidine
Methadone
Propoxyphene
Tramadol
Acetaminophen
Pentazocine
Fentanyl
Salicylates

Sex Hormones
Estogens
Estriol
Estradiol
Estrone
Testosterone
Methyltestosterone
Progesterone
Medroxyprogesterone
Hydroxyprogesterone
Norethindrone
Megesterol

Pituitary Hormones
DDAVP
Methylergonovine

Uterine Hormones
Carboprost
Dinoprostone

Adrenal Steroid Inhibitors
Aminoglutethimide

In one preferred embodiment, the drug is ketoprofen.

Finally, the carrier and drug release agent form a polymeric composition which provides the separate penetration enhancement of facilitating the rapid release of the medication from the cream upon topical application to the patient. The purpose of this combination of materials is to provide for penetration enhancement of a different type than that of the organogel, i.e., by effecting

rapid release of the drug from the cream and transport by the carrier out of the cream and into the skin through the enhanced openings in the stratum corneum.

The drug release agent may be any of a variety of polyoxymers, i.e., polyoxyalkylene derivatives of propylene glycol. Preferred are those which contain mixtures of polyoxyethylene and polyoxypropylene polymeric derivatives of propylene glycol or methyl oxirane polymers. By acting essentially as an emulsifier, stabilizer and dispersing agent, the polyoxymer facilitates the separation of the drug or medication from the other components of the cream and transfers it to the carrier, which will normally be water or a low molecular weight alcohol or organic solvent. Useful polyoxymers are available under the trademark "Pluronic" from Wyandotte Chemical Company.

The concentration of the carrier provided with the drug release agent as a mixture in the cream will determine the particular diffusion coefficient of the drug. With higher concentrations of the carrier, the diffusion coefficient will be lower and the drug will be absorbed more slowly and produce more local effects. Conversely, lowering the concentration of the carrier will speed the absorption of the drug and enhance the ability of the drug to be absorbed systemically. The normal concentration of the drug release agent in the mixture with the cream will be approximately 20% to 30%, with the balance being the carrier, during the formation of the carrier/drug release agent mixture.

The overall concentrations of the various components in the composition will generally be in the ranges of:

Medication	<1%-20%
Solvent for medication	<1%-20%
Organogel	20%-40%
Carrier/release agent	40%-70%

It will of course be understood that these ranges represent the typical ranges for the specific example upon which the Figure is based, i.e., an example with a lecithin organogel, ketoprofen as the drug, and a "Pluronic NF-127" polyoxymer as the drug release agent. In general the ranges for other compositions of this invention in which other suitable organogels, drugs, carriers and release agents are used will be similar, and those skilled in the art will have no difficulty formulating suitable compositions from the description herein.

Other factors will need to be considered in preparing specific formulations. If the carrier concentration in the cream lies above the useful range, it becomes relatively stiff and difficult to apply, or, conversely, if the concentration falls below the suitable level, the cream will have a tendency to separate. Further, the pH of the cream must be adjusted to match the pH of the solubilized medication component to maximize the amount of non-ionized drug present in the cream. All suitable medications have acid/base characteristics that can be altered by adjustment of the pH of the composition. The greater proportion of non-ionized drug present, the greater the drug's solubility and the greater the ability for larger quantities of the drug to be transported transdermally. The control of the pH can also be used to determine whether the drug is likely to become absorbed systemically or to be absorbed locally, since the speed of transdermal transport will be dependent on the pH.

The physical properties of the cream will also be important. As noted the viscosity must be such that it can be applied topically and conform to and adhere to the patient's skin for a period of time sufficient for a significant portion of the medication to be delivered transdermally to the patient. It must also be capable of being removed from the patient's skin with ordinary physiologically acceptable cleansers or solvents, so that the cream may be removed if medically necessary,

or the residue may be removed once the treatment time period for each administration has been completed. The components must be capable of being blended into a smooth, homogenous mixture with a cream- or lotion-like consistency and appearance, which either has a natural light colored appearance or can be lightly tinted if flesh-compatible tones are desired. The cream must also be capable of being covered with a light gauze or other type of dressing if desired, particularly where the cream would otherwise be in contact with the patient's clothing.

Adjustment of pH, effects of concentration and achievement of suitable physical properties in compositions containing polyoxymers have been studied and reported by Chi et al., *J. Pharm. Sci.*, **80** (3): 280-283 (1991). Reference is made to that article, and the prior references reported therein, for guidance in determining practical limits of pH, concentration, viscosity and the like when varying the specific materials herein. The techniques and methods reported there are quite suitable for use in the present invention.

Examples of the formation of different components are given below:

EXAMPLE 1

Formation of a Lecithin Organogel

A number of different lecithin organogels were formed by mixing different quantities of granular lecithin soya with isopropyl palmitate and a solvent . In three different typical compositions the respective amounts of lecithin soya and isopropyl palmitate were 25%/75%, 50%/50%, and 75%/25%. The first composition can be characterized as a thin oil, the second as a medium oil and the third as a heavy oil. In all cases the lecithin granules and isopropyl palmitate were allowed to sit for several hours, commonly overnight, by the end of which a liquid of oil or syrup consistency had formed. Alternatively one can mix the

lecithin soya and the isopropyl palmitate at 50° to 60°C until the dissolution is complete.

At any point during formation of the mixture one can also add the drug or medication. If the latter is soluble in alcohol it may be previously dissolved in the alcohol and the alcohol/drug mixture incorporated into the lecithin soya and isopropyl palmitate mixture.

EXAMPLE 2

Formation of a Carrier/Drug Release Agent Component

A polymeric gel for use as a carrier was formed by mixing 20 grams of a commercial polyoxymer designated as "Pluronic NF-127" with 0.2 g of pure potassium sorbate and adding sufficient refrigerated purified water to bring to volume of 100 ml. Other similar compositions were formed with 30 g and 40 g of the "Pluronic NF-127" respectively. A typical commercial mixer was used to mix the material. Once all of the granules of the polymeric material had been wetted the gel was refrigerated so that dissolution took place upon cooling in the refrigerator. The compositions must be maintained under refrigeration because at ambient conditions they will solidify, since (as opposed to water) polyoxymer mixtures as prepared herein solidify when heated and liquefy when cooled. Stock solutions of these materials may be made and kept in refrigerated storage for repeated use in the formulation of the compositions of the present invention.

EXAMPLE 3

Mixture of a Cream Containing Medication, Lecithin Organogel and Carrier/Drug Release Agent

In a typical procedure equivalent weights of the lecithin soya and the isopropyl palmitate are combined and a small quantity of sorbic acid is incorporated to control pH. The mixture is stirred until a syrup or oil consistency is obtained. Large quantities may be prepared and kept as a stock solution. The

drug or medication, e.g., ketoprofen, is dissolved in water, alcohol or an equivalent solvent by using a the minimal amount of solvent necessary to obtain complete solubilizing. The dissolved drug is added to a small portion of the lecithin organogel and stirred to disperse the drug in the gel. The mixture of the carrier and the polyoxymer is then added to bring the entire formulation to the desired volume, and, if necessary, the pH of the cream is adjusted.

It will be evident that there are numerous embodiments of this invention which, while not expressly described above, are clearly within the scope and spirit of the invention. The above description is therefore intended to be exemplary only, and the scope of the invention is to be limited solely by the appended claims.

WE CLAIM:

CLAIMS

1. A composition useful for diffusional transdermal delivery of medication to a patient, characterized by inclusion of

a. a medication capable of being administered transdermally;

b. a solvent for said medication;

c. a first penetration enhancer which improves diffusion of said medication into and within said patient's skin; and

d. a second penetration enhancer which improves diffusion of said medication out of said composition for transdermal migration;

said composition having a viscosity in a range such that when applied topically, said composition conforms to and adheres to said patient's skin for a period of time sufficient for a significant portion of said medication to be delivered transdermally to said patient.

2. A composition as in Claim 1 wherein said medication is a lipophilic, hydrophilic or amphoteric therapeutic compound.

3. A composition as in Claim 1 or 2 wherein said first penetration enhancer is an organogel and has the property, when applied topically to a patient's skin, of enlarging openings in the stratum corneum, whereby said medication can diffuse through said enhanced openings at a rate greater than its diffusion rate through corresponding unenlarged openings.

4. A composition as in Claim 3 wherein said organogel is a fatty acid phospholipid emulsifying agent and a fatty acid or ester thereof.

5. A composition as in Claim 1 wherein said second penetration enhancer alters the interaction between said medication and said composition to improve diffusion of said medication out of said composition for transdermal migration.

6. A composition as in Claim 5 wherein said second penetration enhancer is a polyoxymer.

7. A composition as in Claim 1 further characterized by including:

a. <1 to 20 parts by weight of said medication capable of being administered transdermally;

b. <1 to 20 parts by weight of said solvent for said medication;

c. 20 to 40 parts by weight of said first penetration enhancer which improves diffusion of said medication into and within said patient's skin; and

d. 40 to 70 parts by weight of said a second penetration enhancer which improves diffusion of said medication out of said composition for transdermal migration.

8. A method for the preparation of a therapeutic composition to be transdermally administered, characterized by inclusion of the following steps:

a. solubilizing a medication capable of being administered transdermally;

b. forming an organogel comprising a first penetration enhancer which improves diffusion of said medication into and within said patient's skin, and a solvent for said solubilized medication;

c. forming a polymeric component comprising a second penetration enhancer which improves diffusion of said medication out of said composition for transdermal migration; and

d. blending said solubilized medication, organogel and polymeric component to form said composition having a viscosity in a range such that when applied topically, it will conform to and adhere to said patient's skin for a period of time sufficient for a significant portion of said medication to be delivered transdermally to said patient.

9. A method as in Claim 8 wherein said medication is a lipophilic, hydrophilic or amphoteric therapeutic compound.

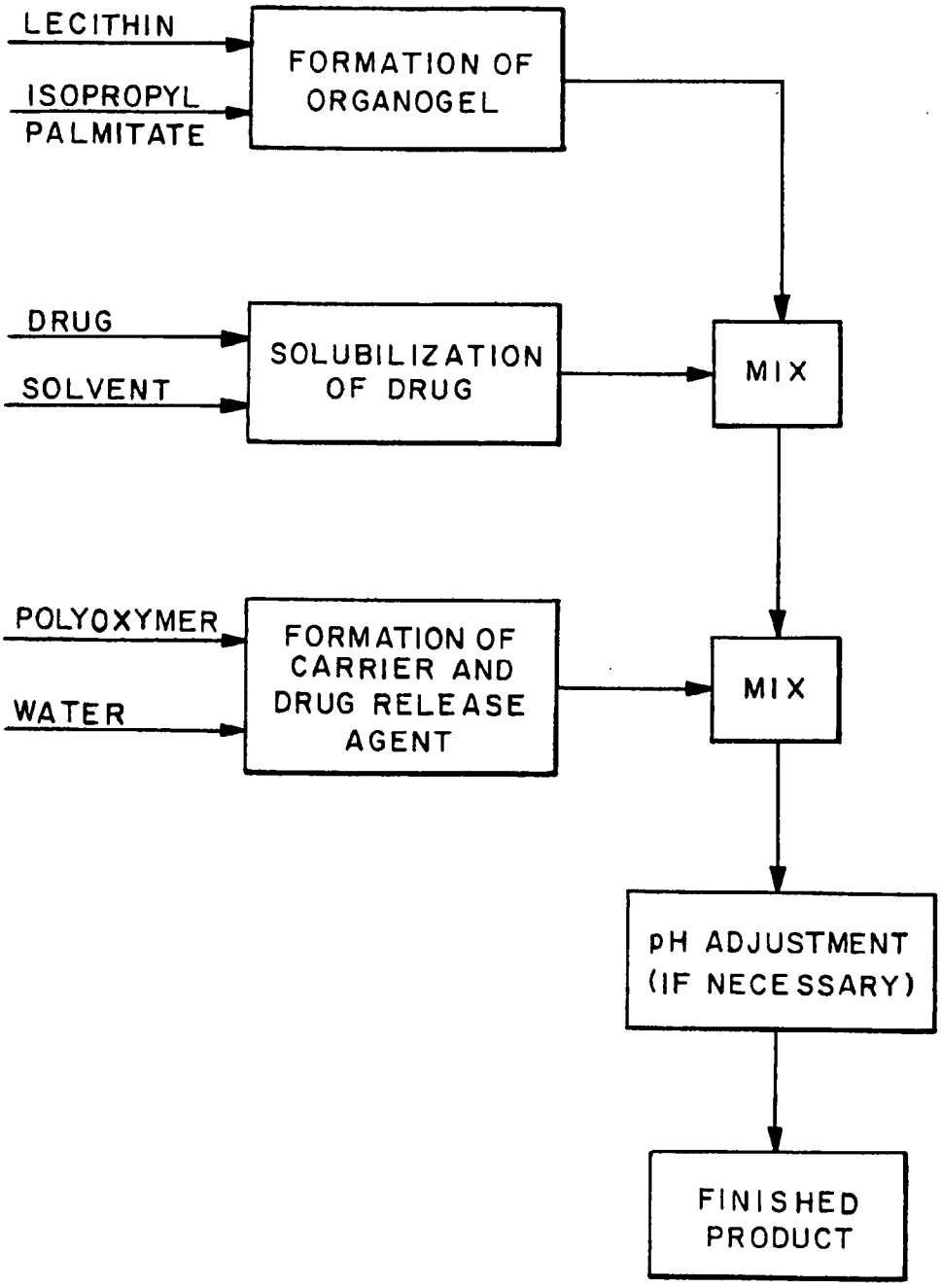
10. A method as in Claim 8 further characterized by:

a. solubilizing <1 to 20 parts by weight of said medication capable of being administered transdermally;

b. forming 20 to 40 parts by weight of said organogel comprising a first penetration enhancer which improves diffusion of said medication into and within said patient's skin, and a solvent for said solubilized medication;

c. forming 40 to 70 parts by weight of said polymeric component comprising a second penetration enhancer which improves diffusion of said medication out of said composition for transdermal migration; and

d. blending said solubilized medication, organogel and polymeric component to form said composition having a viscosity in a range such that when applied topically, it conforms to and adheres to said patient's skin for a period of time sufficient for a significant portion of said medication to be delivered transdermally to said patient.


LECITHIN
ISOPROPYL PALMITATE
FORMATION OF ORGANOGEL
DRUG
SOLVENT
SOLUBILIZATION OF DRUG
MIX
POLYOXYMER
WATER
FORMATION OF CARRIER AND DRUG RELEASE AGENT
MIX
pH ADJUSTMENT (IF NECESSARY)
FINISHED PRODUCT

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 98/23014

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/10 A61K47/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 06636 A (CRANDALL, WILSON) 7 March 1996 see claims 1,3 see page 6, line 23 - page 7, line 1 see page 8, line 6 - line 13 see example 1 ---	1-10
X	US 5 817 699 A (JOHN A. FLORES) 6 October 1998 see claim 1 see column 2, line 9 - line 26 ---	1-10
X	WO 95 11015 A (ELSON, MELVIN) 27 April 1995 see claims 8-12 --- -/--	1-6,8,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1999	02/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ventura Amat, A

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/23014

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 39746 A (ADVANCED POLYMER SYSTEMS) 30 October 1997 see claims 20-23 ---	1-6,8,9
E	WO 99 11208 A (WILLIAMS, C.) 11 March 1999 see claims 1-36 see examples 1-44 -----	1-10

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 98/23014

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9606636 A	07-03-1996	US 5560910 A	01-10-1996
		AU 3417595 A	22-03-1996
		CA 2198362 A	07-03-1996
		EP 0777496 A	11-06-1997
US 5817699 A	06-10-1998	NONE	
WO 9511015 A	27-04-1995	US 5510391 A	23-04-1996
		AU 684850 B	08-01-1998
		AU 8089494 A	08-05-1995
		CA 2174666 A	27-04-1995
		EP 0727985 A	28-08-1996
		JP 9502450 T	11-03-1997
WO 9739746 A	30-10-1997	AU 2804297 A	12-11-1997
WO 9911208 A	11-03-1999	NONE	

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 91/01596
H03K 17/94	A1	(43) International Publication Date:	7 February 1991 (07.02.91)

(21) International Application Number: PCT/CA90/00068

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384,783	25 July 1989 (25.07.89)	US

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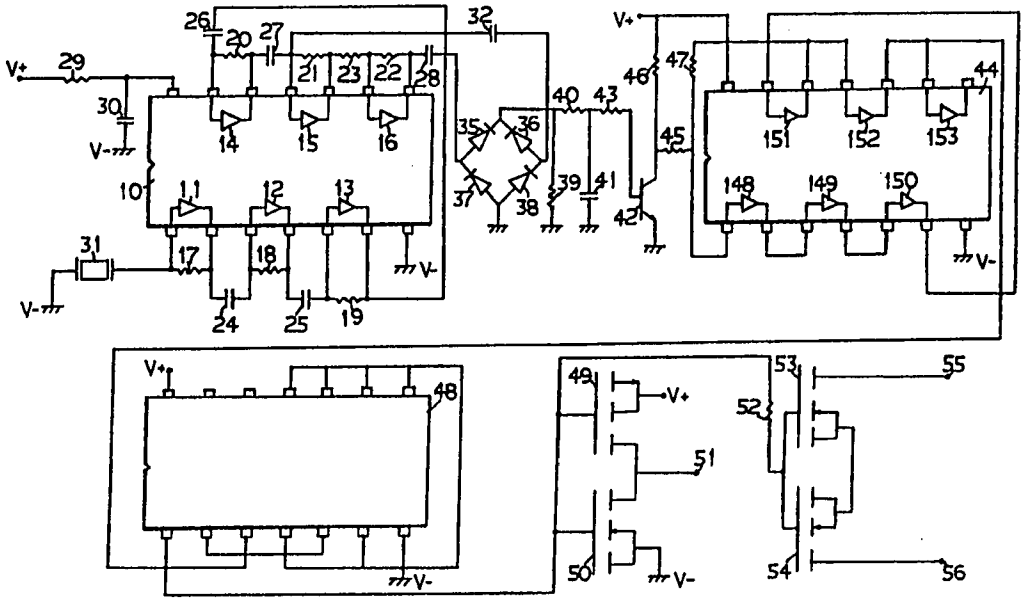
(74) Agent: WONG, David, W.; 46 Willowbrook Road, Thornhill, Ontario L3T 4W9 (CA).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.

(54) Title: ULTRASONIC REMOTE CONTROLLED SWITCHING DEVICE



(57) Abstract

An ultrasonic switching device having very low power consumption is shown. The device is capable of switching high power (55, 56) or generating a power pulse (51) for operating associated electrical control devices. The switching device responses to ultrasonic wave (31) generated by a remote hand-held transmitter unit to change its state from ON to OFF or vice versa and simultaneously generates the desirable power pulse. The remote switching device is suitable for use in remotely controlling the operation of a natural gas fireplace.

* See back of page

DESIGNATIONS OF "DE"

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ULTRASONIC REMOTE CONTROLLED SWITCHING DEVICE

This invention relates to a switching device, and more particularly to an ultrasonic remote controlled switching device having very low power consumption.

Remote controlled switching devices are commonly used for switching electrical appliances such as televisions and the like ON or OFF. The remote controlled switching device may either be incorporated as an integral part of the electrical appliance or is separate from the latter and to which the appliance is connected. The switching devices and the appliances are commonly operated with the household current and they operate with a current draw in excess of 10 milliamperes which, in terms of electronic equipment consumes a relatively large amount of electrical power, particularly when it is necessary for such device to be constantly energized so that it is always ready to be actuated to switch on the equipment of which it controls. Such switching devices are not practical for controlling equipment which are located remote from a household current power source or receptacle. Furthermore, the electrical circuit in such switching devices is usually very complex in construction so that it is costly to manufacture; and the large number of components employed in the circuitry are prone to failure in operation. Attempt to install such switching devices for equipment not readily adjacent to a household current power source may require major alteration of the building structure to route special wiring to supply the operating power to the equipment.

It is the primary object of the present invention to provide a remote controlled switching device having an extremely low power consumption.

It is another object of the present invention to

provide a remote controlled switching device which uses relatively few components which are low cost and is simple and inexpensive to produce.

It is yet another object of the present invention to provide a remote controlled switching device which can be operated with low voltage batteries and yet has a long operating life and can be easily and quickly installed in any desirable location without complicated alteration of the building structure.

It is still another object of the present invention to provide a remote controlled switching device which is operated with ultrasound so that it's operation does not cause any adverse interference to the normal operation of the equipment being controlled or other electrical equipment located in the neighbourhood.

Still yet another object of the present invention is to provide a remote controlled switching device which also generates a power pulse whenever it is actuated for operating associated controlling devices for the equipment being controlled.

Other objects of this invention will appear in the following description and appended claims, reference being made to the accompanying drawings in which:

Figure 1 is an electrical schematic diagram of the switching device according to the present invention.

Figure 2 is an electrical schematic diagram of the ultrasonic sound generating hand-held device usable for remotely actuating the switching device according to the present invention.

Figure 3 is a schematic block diagram showing the use of the remote controlled switching device of the present

invention for operating a gas fireplace.

With reference to the drawings, the remote controlled switching device of the present invention is constructed primarily with three low cost low power consumption metal oxide semiconductors commonly called complementary metal oxide semiconductors or abbreviated as CMOS. Such semiconductors are digital integrated devices and contains a series of inverters. In the present invention, a first digital CMOS device 10 contains six inverters 11 to 16 connected in parallel with resistors 17 to 22 and 23 and output bias and coupling capacitors 24 to 28 to act as cascaded analog amplifiers. The CMOS device 10 can be operated with very low power source such as a 9 volts battery applying a positive potential to the supply pin therein. The operating voltage is supplied through a high resistance resistor 29 in series with the positive potential V+ of the power supply, and is decoupled with a capacitor 30 connected to the negative potential V- of the power supply as shown. The high resistance resistor 29 ensures a very low power consumption by the CMOS device 10, since the supply voltage is automatically adjusted to a low level because of the self-biassing effect provided by the resistor 29. A transducer device 31 such as an ultrasonic transducer is connected to the input of the cascaded amplifiers, so that the signal generated by the transducer 31 is repeatedly amplified by these amplifiers. The low current bias of the amplifiers results in a high output impedance which provides a highly desirable low frequency attenuation. Various types of well known transducers such as an ultrasonic transducer, optical transducer, or electromagnetic wave transducer may be employed which can response to a remotely produced signal to generate a voltage

signal. An ultrasonic transducer is preferred, in that, the sound signal for actuating the transducer is not directional and can reflect from obsticles while it does not cause any adverse interference to the normal operation of any electronic circuit components in other equipment located in the same neighbourhood of the switching device.

The ultrasonic transducer 31 is connected directly to the input of the cascaded amplifiers. The transducer 31 acts as a virtual earth because of the provision of the feedback resistor 17 which is connected from the output to the input of the first amplifier in the cascade. This results in a low input impedance at that amplifier input thus reducing the input noise level.

The amplified signal from the cascaded amplifiers is fed through two coupling capacitors 28 and 32 to a full wave bridge rectifier comprising diodes 35, 36, 37 and 38. Although a full wave rectifier is shown, it can be appreciated that a half wave rectifier may be used instead to provide a similar result. A grounding resistor 39 is connected at the output terminal of the bridge rectifier to the ground such that when the output signal from the rectifier is low or when the output signal is absent, any residual stray charges would bleed through resistor 39 to the ground to ensure no nuisance operation of the device. The series resistor 40 which charges the capacitor 41 is chosen at such value to provide a selected desirable long enough period of time to charge the capacitor 41 to the voltage at which the transistor 42 can be turned on via the resistor 43. As undesirable noise signals are commonly of a short duration, the undesirable noise signals if reaching capacitor 41 will not be of such a level to turn on the transistor 42. Thus, the

transistor 42 can only be turned on by the signal generated by the transducer 31. The transistor 42 as shown in Figure 1 is an NPN transistor. It can be appreciated by those skilled in the art that a PNP transistor may be used for the purpose with the polarity of the diodes of the bridge rectifier connected in the reverse as shown. The collector of the transistor 42 is connected to a second digital CMOS device 44 through coupling resistors 45, 46 and 47. The CMOS device 44 contains six inverters 148,149,150,151,152 and 153 and its terminals are arranged to operate as a Schmidt trigger which converts an input signal into a square wave pulse signal.

The square wave pulse signal from the CMOS device 44 is passed to a third digital CMOS device 48 which is a type D Flip-flop device and is arranged such that its output will change its state from low to high or vice versa whenever a fresh signal is received by its input, namely, whenever, the transducer 31 is actuated by a remote ultrasound generating unit. The output of the CMOS device 48 operating as a Flip-Flop is fed directly to an inverter consisting of a P-channel surface-channel field-effect transistor 49 commonly referred to as P-channel VMOSFET and an N-channel VMOSFET transistor 50. The drain terminal 51 of these VMOSFET transistors 49 and 50 are connected together, so that whenever a signal is generated by the transducer 31 a high power pulse signal appears at the output drain terminal 51 of the VMOSFET transistors 49 and 50. The power pulser may alternatively be constructed with common bipolar NPN or PNP transistors in a voltage follower configuration. Also, single or Darlington transistors may be used for such purpurpose. The output of the Flip-Flop device is also applied via a resistor 52 to one or two N-channel or P-channel

VMOSFET transistors. Two VMOSFET transistors 53 and 54 are shown in the example shown in Figure 1. The output terminals 55 and 56 of the VMOSFET transistors 53 and 54 will change their state from ON to OFF and vice versa whenever a fresh pulse signal is applied from the digital CMOS device 48 to the input of these transistors 53 and 54.

As described above, it can be appreciated that in operation, the switching device of the present invention is represented by the terminals 55 and 56 which will change their states from ON to OFF or vice versa whenever the transducer 31 is actuated by a remote signal and simultaneously a power pulse is present at the drain terminal 51 of the inverter consisting of VMOSFET transistors 49 and 50. Due to the negligible DC current required by the VMOSFET transistors 53 and 54, the operation of the present switching device consumes almost negligible power, in the level of less than 20 microamperes, from the supply voltage power source so that a 9-volt battery can be used as a power source with relatively long operating life.

Figure 2 shows an examplary ultrasound transmitting device which may be used as a remote controlled unit for actuating the switching device of the present invention. Such device may also employ the low power consumption CMOS digital integrated device 57 which contains six inverters 58, 59, 60, 61, 62 and 63. The inverters 58 and 59 are connected to a resistor 64 and capacitor 65 and a potentiometer 66 to provide an oscillator. The actuating power is connected to the input terminal of the CMOS device 57 through the power switch 67 and decoupling capacitor 69. The frequency of the voltage in the oscillator may be selectively varied by adjusting potentiometer 66. The high frequency

voltage signal from the oscillator is then fed through inverters 60 to 63 which act as buffers and output driver amplifiers to drive the piezoelectric wave transducer 68 to generate and emit the ultrasonic wave signal. The simple construction of the transmitting device facilitates low cost manufacturing and it also consumes very low power such that a low voltage battery can be used as a power source with a long operating life.

Due to the relatively small number of components used in the switching device, it can be made in a very compact size and can be freely and easily incorporated into any selected equipment in place of the main ON/OFF switch therein without complex installation. One application of the switching device is in a gas fireplace, as shown in Figure 3, in which the switching device can be easily incorporated in the electromechanical control device 100 of the gas supply to the pilot light 101 and the burner 102 as shown in Figure 3. The electromechanical control device 100 of the gas fireplace is normally actuated with a manual switch 103 which controls the power supply to the electromechanical control device. The manual switch can easily be replaced with the switching device of the present invention or be connected in parallel thereto such that the gas fireplace may be turned ON or OFF remotely with the remote transmitting unit. In the meantime, the power pulse signal generated from the switching device may also be utilized to incorporate with an electromechanical control 104 for controlling the fresh air damper 105 of the gas fireplace, such that whenever the switching device is actuated to turn on the gas fireplace, the power pulse from the switching device will simultaneously operate the electromechanical control

104 to cause the fresh air damper to open so as to allow more air to enter into the fireplace to enhance the ignition of the gas at the burner. The electromechanical control 104 for the fresh air damper of the fireplace may be in the form of a DC motor or solenoid adapted at the damper pivot shaft operative for turning the damper either in one direction or in the opposite direction in response to the polarity of the power pulse.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A low power consumption remote controlled electronic switching device comprising,

a transducer means operative in response to a remote wave signal to generate an electrical signal,

a first digital integrated semiconductor means having a plurality of inverters therein, said inverters being connected in parallel with a plurality of bias resistor means and in combination therewith to form a plurality of amplifier means, said amplifier means being operative to amplify in serial said electrical signal fed from said transducer means to said digital integrated semiconductor means,

rectifier means operative to convert an amplified signal from said amplifier means to a direct current voltage signal,

charging means operative to be charged by said direct current voltage signal,

gating means operative in response to a selected charged voltage level of said charging means to actuate a second digital integrated semiconductor means for generating a square wave signal,

a third digital integrated semiconductor means connected to said second digital integrated semiconductor means and operative to generate a drive signal,

at least one semiconductor means responsive to receiving said drive signal at each instance from said third digital integrated semiconductor means to vary from an existing switch state to an opposite switch state.

2. A remote controlled electronic switching device according to Claim 1 including a second semiconductor means connected to said third digital integrated semiconductor means and operative

in response to receiving a drive signal from said third digital integrated semiconductor means for generating a power pulse.

3. A remote controlled electronic switching device according to Claim 2 wherein said digial integrated semiconductor means are complementary metal oxide semiconductors.

4. A remote controlled electronic switching device according to Claim 3 wherein said semiconductor means are surface-channel field-effect transistors.

5. A remote controlled electronic switching device according to Claim 4 wherein said semiconductor means comprises a pair of surface-channel field-effect transistors each having a base terminal, a collector terminal, an emitter terminal and a drain terminal, said base terminal of said field-effect transistors being commonly connected to an output terminal of said third digital integrated semiconductor means, said emitter terminal and collector terminal of said field-effect transistors being commonly connected together, and said drain terminal of one of said pair of field-effect transistors forming one terminal of said switching device, and said drain terminal of the other field-effect transistor forming a second terminal of said switching device.

6. A remote controlled electronic switching device according to Claim 5 wherein said transducer means is an ultrasonic transducer means operative responsive to remote ultrasonic wave to generate said electrical signal.

7. A remote controlled electronic switching device according to Claim 6 including a remote ultrasonic wave transmitting device comprising a digital integrated semiconductor means having a power input terminal, a low voltage power means connected to said power input terminal through a manually operative switch means, said digital integrated semiconductor means having six inverter

means therein, two of said six inverter means being connected with resistor means to form amplifier means operative for amplifying a power voltage from said voltage power means, an ultrasonic transducer means connected to said digital integrated semiconductor means and operative in response to an amplified signal from said amplifier means to emit an ultrasonic wave signal therefrom.

8. A remote controlled electronic switching device according to Claim 7 including a frequency tuning means connected to said amplifier means and operative to vary the frequency of said ultrasonic wave signal.

9. A remote controlled electronic switching device according to Claim 8 wherein said frequency tuning means includes a capacitor and an adjustable potentiometer connected in parallel to said amplifier means, said potentiometer being adjustable to select the frequency of said ultrasonic wave signal.

10. In a gas fireplace assembly having a burner for providing a flame pattern, a pilot lighter for igniting said burner, and an electromechanical control means for controlling gas supply to said burner and said pilot lighter, including a remote controlled electronic switching device operative for selectively actuating and de-actuating said electromechanical control means, said electronic switching device comprising,

a transducer means operative in response to an ultrasonic wave signal to generate an electrical signal,

a first digital integrated semiconductor means having a plurality of inverters therein, said inverters being connected in parallel with a plurality of bias resistor means and in combination therewith to form a plurality of amplifier means, said semiconductor means having an input terminal connected to said transducer means and operative to amplify in serial said electrical

signal fed from said transducer means to said input terminal,

an operating voltage power means connected to a power terminal of said semiconductor means,

rectifier means connected to an output terminal of said semiconductor means and operative to convert an amplified signal from said amplifier means to a direct current voltage signal,

charge storage means connected to output of said rectifier means and operative to be charged by said direct current voltage signal to a predetermined charge voltage level,

gating means operative in response to a said predetermined charge voltage level of said charge storage means to actuate a second digital integrated semiconductor means for generating a square wave signal,

a third digital integrated semiconductor means connected to said gating means and operative to generate a drive signal,

at least one field-effect transistor means connected to said third digital integrated semiconductor means and operative to receive said drive signal at each instance from said third digital integrated semiconductor means to actuate said electro-mechanical control means selectively.

11. In a gas fireplace assembly according to Claim 10 including a remote ultrasonic wave transmitting device comprising a digital integrated semiconductor means having a power input terminal, a low voltage power means connected to said power input terminal through a manually operative switch means, said digital integrated semiconductor means having a plurality of inverters therein connected in parallel with a plurality of resistor means to form amplifier means, said amplifier means being operative for amplifying a power voltage signal from said voltage power means, an ultrasonic transducer means connected to said digital integrated semiconductor means and operative in response to an

amplified signal from said amplifier means to emit an ultrasonic wave signal therefrom.

12. In a gas fireplace assembly according to Claim 10 or 11 wherein said digital integrated semiconductor means are complementary metal oxide semiconductors.

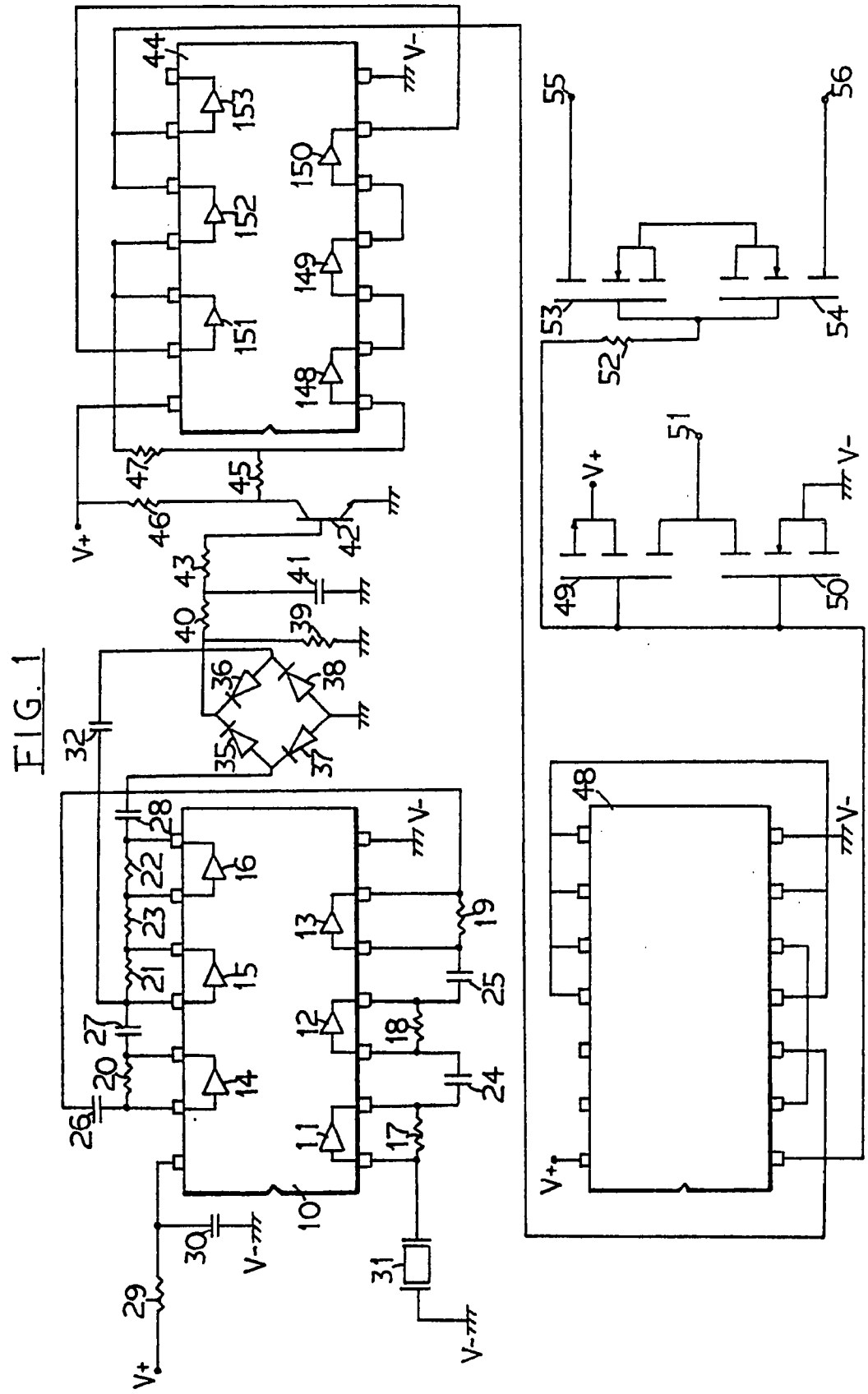
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FIG. 1

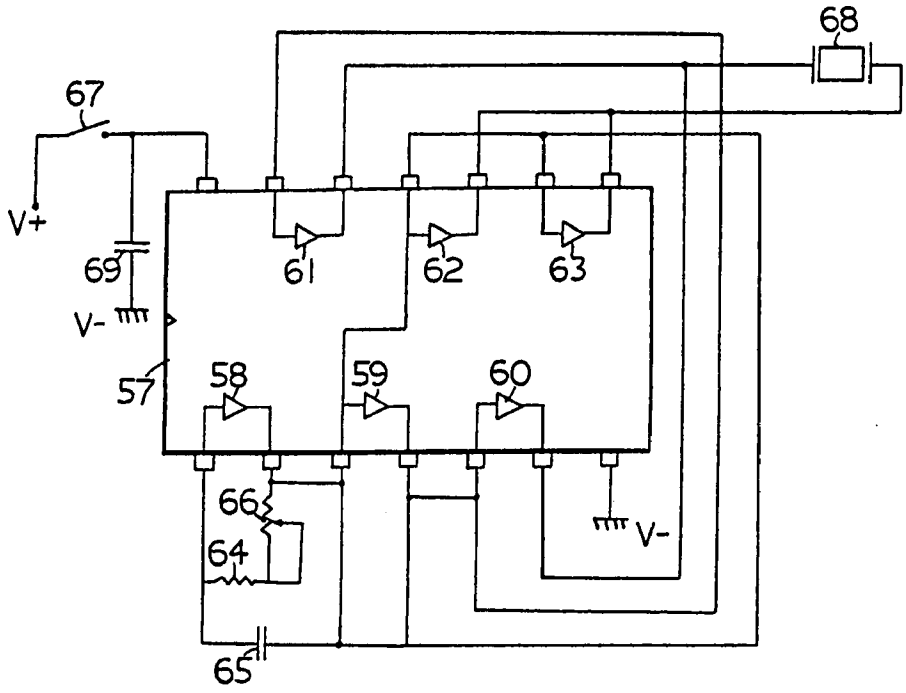
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FIG. 2

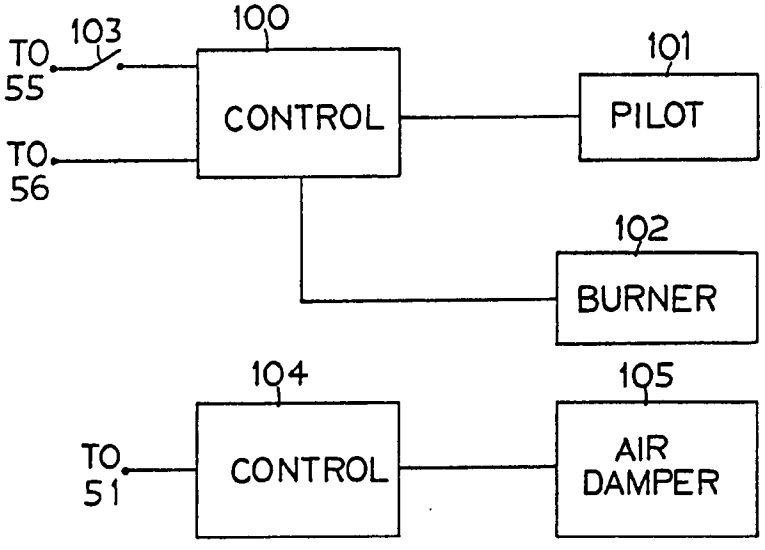
103
100
TO 55
TO 56
CONTROL
101
PILOT
102
BURNER
104
105
TO 51
CONTROL
AIR DAMPER

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00068

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: H 03 K 17/94

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	H 03 K, G 08 C

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 3949366 (SPILLAR et al.) 6 April 1976 see column 2, line 49 - column 4, line 63; figures 2,3	1
Y		2,10
A		6,7
	--	
Y	US, A, 4467224 (MADDOX) 21 August 1984 see column 1, line 61 - column 2, line 61; figure 1	2,10
A		4
	--	
Y	DE, A, 2938734 (SIEMENS AG) 26 March 1981	10

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th June 1990	03 07 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS I. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	see page 7, line 15 - page 8, line 24; figure	
A		11
	--	
A	Elektor, vol. 7, nos. 7/8, July/August 1981, (Canterbury, Kent, GB), "CMOS ultrasonic receiver", page 45, see the whole article	3,12
	--	
A	EP, A, 0265315 (AEROSPATIALE) 27 April 1988 see column 5, lines 6-29; figure 3	5
	--	
A	Elektor, vol. 5, no. 2, February 1979, "Clap-switch", pages 27-29, see page 29, right-hand column, lines 10-28; figure 6	7-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9000068 SA 35241

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3949366	06-04-76	CA-A- 995345	17-08-76
US-A- 4467224	21-08-84	None	
DE-A- 2938734	26-03-81	None	
EP-A- 0265315	27-04-88	FR-A- 2605161	15-04-88
		JP-A- 63102417	07-05-88
		US-A- 4801821	31-01-89